THE

BIOCHEMICAL JOURNAL

CAMBRIDGE UNIVERSITY PRESS
London: FETTER LANE, E.C.
C. F. CLAY, MANAGER

Edinburgh: 100, PRINCES STREET
London: H. K. LEWIS, 136, GOWER STREET, W.C.
WILLIAM WESLEY & SON, 28, ESSEX STREET, STRAND
Paris: LIBRAIRIE HACHETTE & CIE
Berlin: A. ASHER AND CO.
Leipzig: F. A. BROCKHAUS
Chicago: THE UNIVERSITY OF CHICAGO PRESS
Bombay and Calcutta: MACMILLAN AND CO., LTD.
Toronto: J. M. DENT AND SONS, LTD.
Tokyo: THE MARUZEN-KABUSHIKI-KAISHA

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THE

BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

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Volume VII, 1913

Cambridge :
at the University Press
1913

Cambridge:
PRINTED BY JOHN CLAY, M.A.
AT THE UNIVERSITY PRESS

CONTENTS

No. 1 (January)

PAGE

I. The Rate of Protein Catabolism. By Edward Provan Cathcart and Henry Hamilton Green 1

II. The Fate of Indolethylamine in the Organism. By Arthur James Ewins and Patrick Playfair Laidlaw 18

III. The Hydrolysis of Glycogen by Diastatic Enzymes. Comparison of Preparations of Glycogen from different sources. By Roland Victor Norris. (With four figures) 26

IV. The Metabolism of Organic Phosphorus Compounds. Their Hydrolysis by the Action of Enzymes. By Robert Henry Aders Plimmer 43

V. The Hydrolysis of Organic Phosphorus Compounds by Dilute Acid and by Dilute Alkali. By Robert Henry Aders Plimmer 72

VI. The Nitrogenous Constituents of Lime-Juice. By Casimir Funk 81

VII. The Flower Pigments of *Antirrhinum Majus*. I. Method of Preparation. By Muriel Wheldale 87

VIII. The Density and Solution Volume of some Proteins. By Harriette Chick and Charles James Martin 92

IX. A note concerning the Influence of Diets upon Growth. By Frederick Gowland Hopkins and Allen Neville 97

No. 2 (March)

X. The Condensation of Tryptophane and other Indole Derivatives with certain Aldehydes. By Annie Homer 101

XI. On the Colour Reactions of certain Indole Derivatives, and their significance with regard to the Glyoxylic Reaction. By Annie Homer . 116

XII. The Rôle of Glycogen, Lecithides, and Fats in the Reproductive Organs of Echinoderms. By Benjamin Moore, Edward Whitley and Alfred Adams 127

XIII. The Basic and Acidic Proteins of the sperm of *Echinus esculentus*. Direct Measurements of the Osmotic Pressure of a Protamine or Histone. By Benjamin Moore, Edward Whitley and Arthur Webster . . 142

XIV. The Fatty Acids of the Human Brain. By Egerton Charles Grey 148

XV. An Investigation of Phytin. By Robert Henry Aders Plimmer and Harold James Page 157

269995

PAGE

XVI. On the Relations of the Phenols and their Derivatives to Proteins. A contribution to our knowledge of the Mechanism of Disinfection. Part II. A comparative study of the effects of Various Factors upon the Germicidal and Protein-Precipitating Powers of the Phenols. By Evelyn Ashley Cooper 175

XVII. On the Relations of the Phenols and their Derivatives to Proteins. A contribution to our knowledge of the Mechanism of Disinfection. Part III. The Chemical Action of Quinone upon Proteins. By Evelyn Ashley Cooper 186

XVIII. The Rate of Fermentation by Growing Yeast Cells. By Arthur Slator 197

XIX. The Identity of Trimethylhistidine (Histidine-Betaine) from Various Sources. By George Barger and Arthur James Ewins . . 204

XX. A Note on the Metabolism of Nitrogenous Sugar Derivatives. By James Arthur Hewitt 207

XXI. An Attempt to Estimate the Vitamine-Fraction in Milk. By Casimir Funk 211

XXII. The Enzymes of Washed Zymin and Dried Yeast (Lebedew). I. Carboxylase. By Arthur Harden 214

No. 3 (May)

XXIII. The Critical Solution Point of Urine. By William Ringrose Gelston Atkins and Thomas Arthur Wallace. (With one diagram) . 219

XXIV. Quantitative Relations in Capillary Analysis. By Hans Schmidt. (With three diagrams) 231

XXV. A Note on the Hopkins and Cole Modification of the Adamkiewicz Test for Protein. By Vernon Henry Mottram 249

XXVI. The Use of Litmus Paper as a Quantitative Indicator of Reaction. By George Stanley Walpole 260

XXVII. The Preparation from Animal Tissues of a Substance which cures Polyneuritis in Birds induced by Diets of Polished Rice. Part I. By Evelyn Ashley Cooper 268

XXVIII. On the Lipolytic Action of the Blood. By Francis Hugo Thiele 275

XXIX. On the Lipolytic Action of the Tissues. By Francis Hugo Thiele 287

XXX. The Estimation of Tyrosine in Proteins by Bromination. By Robert Henry Aders Plimmer and Elizabeth Cowper Eaves . . 297

XXXI. The Separation of Cystine and Tyrosine. By Robert Henry Aders Plimmer 311

XXXII. The Factors concerned in the Solution and Precipitation of Euglobulin. By Harriette Chick 318

No. 4 (July)

PAGE

XXXIII. The Fat of Yeast. By Allen Neville 341

XXXIV. The Influence of the Carbonates of the Rare Earths (Cerium, Lanthanum, Yttrium) on Growth and Cell-Division in Hyacinths. By William Howel Evans. (With Plate) 349

XXXV. On the Chemical Nature of Substances from Alcoholic Extracts of Various Foodstuffs which give a Colour Reaction with Phosphotungstic and Phosphomolybdic Acids. (Preliminary Communication.) By Casimir Funk and Archibald Bruce Macallum 356

XXXVI. The Production of Acetaldehyde during the Anaerobic Fermentation of Glucose by *Bacillus coli communis* (Escherich). By Egerton Charles Grey 359

XXXVII. The Biochemical Synthesis of the Fatty Acids. By Ida Smedley and Eva Lubrzynska 364

XXXVIII. The Condensation of Aromatic Aldehydes with Pyruvic Acid. By Eva Lubrzynska and Ida Smedley 375

XXXIX. The Precipitation of Egg-Albumin by Ammonium Sulphate. A Contribution to the Theory of the "Salting-out" of Proteins. By Harriette Chick and Charles James Martin. (With six figures) . 380

XL. Some Observations on the Estimation of Urea. By John Alexander Milroy 399

XLI. Gas-Electrode for General Use. By George Stanley Walpole. (With four figures) 410

XLII. Some Esters of Palmitic Acid. By Marjory Stephenson . 429

No. 5 (October)

XLIII. Hydrolysis of Proteins with an Alcoholic solution of Hydrogen Chloride. Part I. By Charles Weizmann and Ganesh Sakharam Agashe 437

XLIV. The Flower Pigments of *Antirrhinum Majus*. II. The pale yellow or ivory pigment. By Muriel Wheldale and Harold Llewellyn Bassett 441

XLV. Observations on the use of the Folin method for the estimation of Creatine and Creatinine. By William Henry Thompson, the late Thomas Arthur Wallace and Harold Reginald Septimus Clotworthy . . 445

XLVI. Note on the iodine content of fish-thyroids. By Alexander T. Cameron 466

XLVII. The Combinations of Haemoglobin with Oxygen and with Carbon Monoxide. I. By Archibald Vivian Hill 471

PAGE

XLVIII. The Combinations of Haemoglobin with Oxygen and with Carbon Monoxide. II. By Joseph Barcroft. (With five figures) . 481

XLIX. Separation of Proteins. Part III. Globulins. By Henry Cobden Haslam 492

L. The Chemistry of the Leucocytozoon Syphilidis and of the host's protecting cells. By James Eustace Radclyffe McDonagh and Robert Lauder Mackenzie Wallis 517

No. 6 (December)

LI. Enzyme-action, Facts and Theory. By Hendrik Pieter Barendrecht. (With two figures) 549

LII. An Investigation into the Physico-chemical Mechanism of Haemolysis by Specific Haemolysins. (Preliminary Communication.) By Upendra Nath Brahmachari 562

LIII. Notes on some further Experiments on the Clotting of Caseinogen Solutions. By Samuel Barnett Schryver 568

LIV. On the Cholesterol Content of the Tissues of Cats under various Dietetic Conditions and during Inanition. By John Addyman Gardner and Percy Edward Lander 576

LV. On the Oxidation of Coprosterol and Coprostanone. Part I. By John Addyman Gardner and William Godden 588

LVI. A note on a Modification of Teichmann's Test for Blood. By Claude Trevine Symons 596

LVII. On the Behaviour of Amylase in the Presence of a Specific Precipitate. By Agnes Ellen Porter 599

LVIII. The Galactosides of the Brain. I. By Otto Rosenheim . 604

LIX. The Estimation of Pyruvic Acid. By Ida Smedley MacLean 611

LX. Note on Isocholesterol, Coprosterol and the Classification of the Sterols. By Charles Dorée 616

LXI. The Hydrolysis of Glycogen by Diastatic Enzymes. II. The influence of Salts on the Rate of Hydrolysis. (Preliminary Communication.) By Roland Victor Norris. (With one figure) 622

LXII. The Enzymatic Formation of Polysaccharides by Yeast Preparations. By Arthur Harden and William John Young 630

Index 637

I. THE RATE OF PROTEIN CATABOLISM[1].

By EDWARD PROVAN CATHCART and HENRY HAMILTON GREEN.

Physiological Laboratory, Glasgow University.

Received November 9th, 1912.

The whole question of the course of the breakdown of the protein molecule within the tissues is one of the most obscure in physiology. We have now a fairly good idea of the form in which the protein is absorbed from the lumen of the intestine but its immediate fate is still unknown. Apparently however definite evidence is now collecting [Folin, 1912] in support of the view that no immediate synthesis, analogous to that of fat, takes place. Unquestionably, irrespective of the form in which the protein material is conveyed to the tissues, there is, soon after the ingestion of food, a fairly complete disintegration of the protein molecule as evidenced by the increase in the output of nitrogenous substances in the urine. Of course it might be maintained, as it has been, that this material arises not from the newly ingested material but from "effete" protoplasm broken down and discarded when a new supply of repair material is available. If this be so then the material excreted ought to bear some definite percentage relationship to the normal protein of the body, for example the ratio of sulphur to nitrogen in the urine should approximate to that of the average tissue as obtained say by the study of the sulphur and nitrogen ratio in complete starvation.

It was thought that, by a careful study of the ratios of S : N after feeding with specially chosen foodstuffs, light might be thrown on :—

(1) The rate of protein catabolism.

(2) The nature of the material catabolised.

Previous Work.

Little work has been carried out in this field probably because of the fact that until recently the difficulty of carrying on a long series of sulphur analyses was considerable. Since the introduction of the very excellent and rapid method of S. R. Benedict this difficulty has largely disappeared.

[1] The majority of the results here published were given in the form of a communication at the Biochemical Society's Cambridge meeting, October 1911.

Siven [1901] found that the sulphur output ran closely parallel to the nitrogen output. He came to the conclusion that all the nitrogen excreted does not come from a purely protein source.

Sherman and Hawk [1901] also found that the nitrogen and sulphur outputs ran very closely parallel.

Von Wendt [1905] made a very thorough investigation into the output of both nitrogen and sulphur. He found that when protein was broken down in the body, the sulphur-rich digest products were the first to be catabolised, i.e. that the sulphur output preceded that of the nitrogen and that therefore the nitrogenous products which were retained in the tissues were comparatively poor in sulphur. Von Wendt maintained that it is only when the nitrogen and sulphur excretions are considered together that the true picture of the total protein exchange in the body can be obtained: individually considered they only tell whether certain decomposition products are excreted.

Ehrström [1906] pointed out that, although the nitrogen and sulphur excretions ran practically parallel, the sulphur output acted more rapidly with any change in the intake. He suggested that the sulphur-containing amino-acids were more readily oxidisable in the organism and that this might account for the accelerated rate of output of the sulphur in comparison with that of the nitrogen.

Falta [1906] also supported the view that the protein molecule was catabolised in a step-like fashion. He believed that certain nuclei were more labile than others and that this lability would account for the appearance of certain products before other constituent substances of the protein molecule. He further pointed out in a very clear way that even in the case of the nitrogen-containing moiety of the protein molecule the output of the excess nitrogen or waste nitrogen was not immediate but that it might—depending on the nature of the material ingested—be spread over several days. Thus after the ingestion of caseinogen an amount of nitrogen equivalent to that taken in was excreted in four days whereas after the administration of egg albumin the excretion was continued to the sixth day.

Hämäläinen and Helme [1907] as a result of their experiments with a superimposed diet as used by Falta came to the conclusion that the output of sulphur ran very closely parallel to the output of nitrogen. At the same time they held that their results bore out fully the contention of von Wendt that the sulphur-rich products arising from the catabolism of the protein molecule are more rapidly burnt and excreted than those which are sulphur-poor.

Since our work was completed, two papers by Wolf [1912] have appeared which practically cover the same ground as our own. Wolf found that in some instances the maximum of the output of sulphur preceded that of the nitrogen but that in others the sulphur appeared simultaneously with or was behind the nitrogen. He arrived at the general conclusion however that in the majority of instances the sulphur part of the protein molecule is the site of the first catabolic process.

Methods.

In our experiments we also adopted the superimposition method of feeding introduced by Falta, i.e. we added on a particular day to a standard diet the special foodstuff the rate of catabolism of which we wished to establish. The standard diet was always started four or five days previous to the test in order to get the organism into a state of approximate equilibrium. (It may be remarked here that in the majority of instances, particularly those on a low protein intake, the lowest level of nitrogen was probably never reached.) On a set day there was then added to the ordinary diet, as a rule with the morning meal, the special foodstuff. Then on the following two, three or four days the original diet was continued without addition of any kind. As far as possible throughout the feeding period the intake of fluid was kept constant. The subject H. H. G. lived an ordinary quiet laboratory life, taking no undue exercise.

The diets which we employed were (1) a low nitrogen diet consisting of tapioca and cream or potatoes and butter; (2) a diet containing a medium amount of nitrogen made up of bread, butter and milk and finally (3) a diet rich in nitrogen consisting of eggs, cheese, milk and bread.

As we were only concerned with the rate at which the nitrogen and sulphur were excreted the output of total nitrogen and total sulphur were alone considered. As a general rule, as the diets were creatine- and creatinine-free, the opportunity was taken of studying the output of these substances under the different conditions of the experiments. The total nitrogen was estimated by the ordinary Kjeldahl method and the total sulphur by Benedict's method [1909], creatine and creatinine by Folin's method.

In order to get as perfect a picture as possible, the outputs of total nitrogen and of total sulphur were estimated every two hours during the day of the experiment. As a rule, for purposes of comparison the estimations on the preday, and sometimes on the day immediately following, were also carried out on the two-hourly plan. The output of the total nitrogen in the faeces was also determined.

GELATIN.

Three experiments were carried out with gelatin superimposed on various diets, (1) tapioca, (2) potato diet, (3) the egg diet.

The first experiment was carried out with the tapioca diet which had been given for two days previously. The amount of gelatin given was that present in 515 grams of a "table jelly," which contained seven grams of nitrogen and ·503 grams of sulphur, with therefore an S:N ratio of 1:13·9. The whole of the gelatin was taken with the breakfast meal at 9 a.m. The following table gives the result of the experiment.

TABLE I.

Gelatin feeding (tapioca diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N:S ratio	Creatinine in grms.	Remarks
Sept. 16, 1910	9·78	·624	15·7	2·02	
,, 17, ,, 9–11	·568	·0356	16·0	·162	Two-hourly collection.
11– 1	·554	·0384	14·4	·168	
1– 3	·901	·0462	19·5	·181	
3– 5	·697	·0366	19·0	·162	
5– 7	·618	·0385	16·0	·177	
7– 9	·680	·0549	12·4	·182	
9–11	·506	·0292	17·5	·164	
11– 9	2·050	·1366	15·0	·718	
	6·574	·4160	15·8*	1·92	
Sept. 18, 1910, 9–11	·490	·0354	13·8	·170	Fed day.
11– 1	·643	·0505	12·7	·178	515 grms. "table jelly'
1– 3	·902	·0735	12·3	·177	=7 grms. N taken at
3– 5	·697	·0607	11·5	·166	9 a.m.
5– 7	·645	·0658	9·8	·166	
7– 9	·918	·0594	15·5	·187	
9–11	·838	·0395	21·2	·160	
11– 9	2·732	·1389	19·7	·693	
	7·865	·5237	15·0*	1·90	
Sept. 19, 1910, 9–11	·462	·0291	15·9	·162	
11– 1	·539	·0352	15·3	·180	
1– 3	·566	·0311	18·2	·170	
3– 5	·574	·0284	20·2	·168	
5– 7	·454	·0256	17·7	·162	
7– 9	·798	·0411	19·4	·176	
9–11	·532	·0286	18·0	·168	
11– 9	2·197	·1254	17·5	·706	
	6·122	·3445	17·7*	1·90	
Sept. 20, 1910	5·7	·3189	18·1	1·90	

* Ratio of totals.

When the two-hourly outputs of the preday and the fed day are compared it will be noted that in the case of the total nitrogen the alteration from the preday takes place late in the afternoon whereas with the sulphur the change occurs a few hours after the ingestion of the gelatin. From an examination of the S : N ratios the increased rate of the sulphur output over the nitrogen is clearly seen. Even at the end of the first two hours the ratio of sulphur to nitrogen has increased to 13·8 as compared with the ratio of the total outputs of the previous day of 15·8. As will be noted the output of sulphur accelerated over that of the nitrogen until the ratio stood at 9·8. Later on however the rate of the output of nitrogen suddenly increased with the result that the apparent lag was almost done away with, the ratio of S : N falling to 21·2. This gives an average for the total outputs of the sulphur and the nitrogen for the day of 15·0, a result but little different from the preday. The postday shows a fall to 17·7. As regards the total output of the superimposed nitrogen and sulphur there is evidence of a marked retention of both substances. Of the ingested nitrogen only 18·6 % is excreted and of the sulphur 26·2 %. On neither of the two postdays is there any evidence of the excretion of the retained nitrogen, 5·7 grams.

Unfortunately no estimation of the nitrogen excreted by way of the faeces was carried out as previous experiments with other forms of superimposed foodstuffs had shown that no marked excretion took place by that channel. Still the retention here might have been only apparent, i.e. the bulk of the nitrogen might have been excreted by way of the faeces. Of course at the same time it was possible that a true retention of nitrogen had taken place especially as the subject was on a tapioca diet where a special demand for protein might be presumed.

In order to test this possibility the gelatin in the following experiment was superimposed on a diet rich in protein. The diet consisted of 10 eggs, 4 ozs. of cheese, 2 pints of milk, 8 ozs. of bread and 3 ozs. of butter. The gelatin employed was the same as in the previous experiment and the same amount was given. The only difference was that the subject had so much difficulty in consuming it with his otherwise abundant morning meal that about one third had to be left till the midday meal. Again, in order to get nitrogen equilibrium on the diet previous to the superimposition there were six predays of feeding (the urine on the sixth day being collected two-hourly). As will be seen from the following table the effect of the superimposition was not that of the previous experiment. Here there is neither a marked rise in the output of either the nitrogen or the sulphur nor is there an increase in the S:N ratio; indeed there is a slight decrease.

TABLE II.

Gelatin feeding (egg and cheese diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Remarks
Oct. 9, 1911	16·0	1·231	13·0	—	Weight 70·7 kilos.
„ 10, „	17·0	1·270	13·4	—	„ 70·9 „
„ 11, „	16·7	1·292	13·0	1·97	„ 71·0 „
„ 12, „ 9–11	1·46	·1091	13·4	·165	„ 71·6 „
11– 1	1·40	·0996	14·0	·178	Two-hourly collection.
1– 3	1·54	·1036	14·8	·176	
3– 5	1·48	·1042	14·2	·178	
5– 7	1·46	·1041	14·1	·176	
7– 9	1·76	·1295	13·6	·178	
9–11	1·58	·1315	12·0	·165	
11– 9	6·93	·5720	12·1	·755	
	17·61	1·349	13·0*	1·97	
Oct. 13, 1911, 9–11	1·55	·1179	13·2	·170	Weight 71·2 kilos.
11– 1	1·66	·1266	13·1	·174	515 grms. "table jelly"
1– 3	1·91	·1259	15·2	·168	with 7 grms. N taken ⅔
3– 5	1·90	·1213	15·7	·176	at 9 a.m., ⅓ at 1 p.m.
5– 7	1·89	·1135	16·7	·178	
7– 9	1·92	·1122	17·1	·183	
9–11	1·63	·1136	16·9	·179	
11– 9	7·82	·5793	13·5	·736	
	20·28	1·410	14·4*	1·96	
Oct. 14, 1911, 9–11	1·61	·120	13·3	·179	Weight 71·6 kilos.
11– 1	1·69	·113	14·9	·182	
1– 3	1·62	·110	14·7	·179	
3– 5	1·55	·112	13·0	·184	
5– 7	1·52	·112	12·7	·176	
7– 9	1·88	·143	13·1	·180	
9–11	1·20	·133	10·9	·176	
11– 9	7·60	·654	11·6	·775	
	18·67	1·497	12·4*	2·03	
Oct. 15, 1911	19·0	1·448	13·1	2·04	
„ 16, „	18·6	1·389	13·4	1·99	
„ 17, „	18·8	1·392	13·5	1·97	Weight 71·6 kilos.

* Ratio of totals.

If the normal output of nitrogen for the predays be taken as 17·1 gram, the mean of the three predays preceding the superimposition, then practically the whole of the ingested extra nitrogen was excreted at the end of the second day after the feeding. 45·4 % of the extra nitrogen was excreted on the day of feeding, 22·4 % on the following day and 27·1 % on the second day after. For some inexplicable reason the output of nitrogen does not return to its old level.

In this experiment the output of nitrogen in the faeces was followed. The average output for four predays (collected and estimated each day) was 3·13 grams. The output on the day of feeding was 3·15 grams, on the first postday 3·72 grams, and the average daily output for the three following days was 2·59 grams. Evidently then the retention of the nitrogen in the first experiment was due to the actual retention of the nitrogen within the tissues and not to an excretion by way of the intestine.

As regards the output of the sulphur as already noted there is a slight fall in the ratio on the day of feeding to be followed however on the first postday by a slight rise; on the other postdays the ratio practically returns to the normal ratio found previous to the day of feeding.

If the normal output of sulphur be taken as the average of the four predays, viz. 1·31 gram, then on the day of feeding 21·8 % of the ingested sulphur has been excreted, 35·8 % on the first post-day, and 29·8 % on the second. Thus the whole of the ingested sulphur, just as in the case of the nitrogen, is excreted within three days but also, just as with the nitrogen, instead of returning to its original level the output of sulphur continues slightly above normal. It is extremely difficult to offer any adequate explanation for this continued increase in the outputs of nitrogen and sulphur unless it be that the ingestion of the gelatin has stimulated protein catabolism.

Later a third experiment was tried in which there was a return to a lower nitrogen intake. The diet on this occasion consisted of boiled rice 10 ozs. (weighed dry), potatoes 1 lb. (weighed after peeling), butter 6 ozs., milk ½ pint, containing about 5·5 grams nitrogen. The day of superimposition was preceded by six days on the diet alone, the food being taken in four meals. On the seventh day of the diet 670 grams of a "table jelly" were superimposed. The subject found it absolutely impossible to consume this amount of gelatin at the first meal, and accordingly half the amount was taken at breakfast, one fourth at midday, and the other fourth with the afternoon meal. No attempt was made in this experiment to collect the urine two-hourly. The faeces were again examined for their output of nitrogen and the following figures show quite conclusively that no loss took place by this channel: average daily output on the predays 3·24 grams, day of feeding 3·22 grams, on days immediately following 3·12 grams and 3·24 grams.

As regards the output of nitrogen in the urine it is found that there is an immediate rise on the day of feeding and that there is also a distinct increase in the output during the three following days. The same statement holds

TABLE III.

Gelatin feeding (rice, potatoes, butter and milk diet).

Date	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Weight, kilos.	Remarks
March 19, 1912	8·53	·658	12·9	—	—	
20	7·82	·678	11·6	—	72·2	
21	7·02	·556	12·4	1·94	72·3	
22	6·85	·582	12·3	1·92	—	
23	6·72	·547	11·7	1·92	72·0	
24	6·60	·564	11·7	1·92	71·8	
25	10·18	·883	11·4	1·91	—	On the 25th 670 grms. "table
26	9·07	·663	13·7	1·96	72·0	jelly" taken (= 9·12 grms. N)
27	7·60	·663	11·5	1·94	—	½ at 8.30 a.m., ¼ at 1.30 p.m.
28	7·14	·582	12·3	—	—	& ¼ at 5p.m. Urine collected
29	6·60	·560	11·8	—	71·9	from 8.30 a.m. to 8.30 a.m.

good for the output of sulphur during these days. There is a curious irregularity in the S : N ratio which cannot be accounted for. The results were all checked by repeat analyses and found to be correct.

As regards the percentage amount of the ingested nitrogen and sulphur which was excreted during the day of feeding and the three following days the table below gives the result very clearly.

TABLE IV.

	N	S
	Percentage excreted	
Fed day	37·9	59·0
First day after	25·7	18·5
Second ,, ,,	9·6	18·5
Third ,, ,,	4·6	3·9
	77·8	99·9

Thus it will be noted that there is again quite a well-marked retention of nitrogen although it does not take place in the case of the sulphur. This retention is not so conspicuous as that which took place in the first experiment. The difference in result may be due to the fact that in the second experiment although the protein content is comparatively low still the diet is a more mixed one. Both experiments demonstrate that the sulphur, as other observers have previously found, is excreted more rapidly than the nitrogen.

Egg Albumin.

Two experiments were carried out with boiled egg-white superimposed in the first case on the tapioca diet and in the second on the potato and butter diet.

In the first experiment the tapioca was given for two days previously, the urine on the second day being collected two-hourly. On the third day 8 ozs. of boiled egg-white containing 4·75 grams nitrogen and ·594 gram sulphur, thus with a S : N ratio of 1 : 8, were taken with the first meal. The collection of the urine was continued on the tapioca diet for still another day. The results obtained are given in the following table.

TABLE V.

Egg-albumin feeding (tapioca diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Remarks
Aug. 18, 1911	8·81	—	—	—
„ 19, „ 9–11	·68	·0374	18·0	Two-hourly collection.
11– 1	·64	·0394	16	
1– 3	·63	·0344	19	
3– 5	·83	·0436	19	
5– 7	·78	·0428	18	
7– 9	·72	·0507	14	
9–11	·38	·0266	14	
11– 9	2·17	·1317	16	
	6·83	·4066	17*	
Aug. 20, 1911, 9–11	·344	·0266	13	225 grms. boiled egg-white (=4·75 grms. N) superimposed at 9 a.m.
11– 1	·406	·0340	12	
1– 3	·589	·0437	13·5	
3– 5	·813	·0710	11·4	
5– 7	·675	·0580	11·7	
7– 9	·874	·0827	10·6	
9–11	·788	·0691	11·4	
11– 9	2·070	·1870	11·1	
	6·56	·5721	11·4*	
Aug. 21, 1911, 9–11	·399	·0392	10·2	
11– 1	·382	·0416	9·2	
1– 3	·580	·0419	14·0	
3– 5	·473	·0409	11·5	
5– 7	·468	·0353	13·3	
7– 9	·474	·0366	13·0	
9–11	·672	·0425	15·8	
11– 9	2·090	·1356	15·4	
	5·54	·4136	13·4*	

* Ratio of totals.

It will be noted that the total output of nitrogen is actually less on the fed day although there is an increase in the output of sulphur. When the two-hourly outputs of the preday and the feeding day are compared it is seen that the diminution in the output of the nitrogen is due to some

apparent retention during the early hours of the day. Although the rise takes place on both days about the eighth hour, on the fed day instead of a diminution following there is a marked increase, particularly at the twelfth and fourteenth hours. During the night period however there is less nitrogen excreted than during the night of the preday.

As regards the sulphur output on the fed day there is also less sulphur excreted during the first four hours than during the same period on the preday but thereafter there is a well-marked rise in the output. This acceleration in the rate of the output of sulphur is particularly noticeable when the S:N ratios are compared with those of the preday. The actual maximum on the preday is 14, whereas on the fed day it rises to 10·6.

There is no rise in the output of nitrogen on the day following although the output of sulphur still shows some increase.

In this experiment it is clear that under the conditions employed there has been a complete retention of the 4·75 grams of nitrogen given, but, of the ·594 gram sulphur, ·173 gram has reappeared, i.e. 29 % of the amount ingested.

A second experiment was carried out in which 11 ozs. of boiled egg-white containing 6·5 grams nitrogen and ·81 gram sulphur with a S:N ratio of 1:8 were superimposed on a potato and butter diet containing about 4 grams of nitrogen (3 lbs. potatoes and ½ lb. of butter). In this experiment four days of the diet were carried out and then the egg-white was taken with the morning meal on the fifth day. The diet was continued and the urine collected for two days more.

The results will be found in Table VI (page 11).

Here it will be noted that there is not a complete retention of the ingested nitrogen although as in the previous experiment there is quite a well-marked rise in the output of the sulphur. When the two-hourly outputs of the fed day and the preday are compared it is found that the outputs of nitrogen bear a very close resemblance to one another. In each the maximum output takes place about the eleventh and twelfth hours. In the case of the sulphur the output rapidly rises and continues well above the preday throughout. This accelerated output of sulphur is also clearly demonstrated when the S : N ratios are examined. The maximum ratio on the preday is 10 whereas on the fed day it rises to 7·6, the total ratio for this day compared with that of the preday being 9·8 to 12.

On the day following there is no continued rise in the output of nitrogen and the sulphur output also falls back practically to the preday level, although the S : N ratio is still high, 10·7, indicating a continued acceleration

in the output of the sulphur. The second day after the feeding shows a further fall in the output of nitrogen and also in that of the sulphur. The S : N ratio is however still high, being 10.

TABLE VI.

Egg-albumin feeding (potatoes and butter diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Remarks
Sept. 3, 1911	9·8	—	—	—	
„ 4, „	8·3	·804	10·4	2·06	
„ 5, „	7·6	·685	11·1	1·97	
„ 6, „ 9–11	·700	·0668	10·5	·174	
11– 1	·767	·0688	11·1	·182	
1– 3	·526	·0536	10·0	·170	
3– 5	·743	·0597	12·4	·180	
5– 7	·637	·0626	10·2	·180	
7– 9	·980	·0653	15·0	·182	
9–11	·764	·0584	13·1	·186	
11– 9	3·100	·2482	12·5	·850	
	8·217	·6834	12·0*	2·10	
Sept. 7, 1911, 9–11	·768	·0604	12·6	·177	312 grms. boiled egg-white (=6·5 grms. N) superimposed at 9 a.m.
11– 1	·788	·0653	12·1	·171	
1– 3	·663	·0664	10·0	·170	
3– 5	·786	·0974	8·1	·181	
5– 7	·786	·1027	7·6	·173	
7– 9	1·037	·0933	11·0	·173	
9–11	·781	·0871	9·0	·169	
11– 9	3·394	·3436	9·9	·737	
	9·011	·9162	9·8*	2·00	
Sept. 8, 1911, 9–11	·722	·0612	11·8	·168	
11– 1	·755	·0728	10·4	·178	
1– 3	·636	·0584	9·2	·176	
3– 5	·522	·0538	9·7	·174	
5– 7	·593	·0608	9·7	·182	
7– 9	·805	·0755	10·6	·188	
9–11	·590	·0584	10·1	·169	
11– 9	2·721	·2442	11·1	·734	
	7·344	·6851	10·7*	1·97	
Sept. 9, 1911	6·70	·676	10·0	1·97	

* Ratios of totals.

Thus of the 6·5 grams of nitrogen taken in (the mean of the three pre-days' output of nitrogen being 8·01 grams and of the sulphur for two days ·684 gram) there has only been 1 gram or 15·3% excreted and that on the day of feeding. Of the sulphur ·232 gram or 28·6% was excreted on the day of feeding and a mere trace on the first day after.

These results do not agree very well with those obtained by Wolf. He found that the sulphur output lagged behind that of the nitrogen. He had previously observed the same curious inversion of the output rates in his feeding experiments with uncoagulated egg albumin. This delay in the output of the sulphur did not take place however when predigested egg albumin was fed; in this case the sulphur output preceded that of the nitrogen.

We had also carried out a couple of experiments with uncoagulated egg albumin but we had used instead of the ordinary raw material from eggs a finely powdered dry product (Egg albumin extra fine powder, Merck). In both experiments the albumin was superimposed on a tapioca diet. As these experiments were amongst our earliest they were not so thoroughly carried out as those previously described. In the first, in which 6·7 grams of nitrogen were given, there was a retention of at least 5·3 grams nitrogen. (The sulphur output was not measured.) In the second experiment, in which the same amount of nitrogen was given, there was a retention of about 6·0 grams. In neither case was the urine collected on any day following the feeding. As regards the output of sulphur in the second experiment the total amount excreted rose above that of the preday, the S : N ratios altering from 14·3 on the preday to 11·5 on the day of feeding. In this experiment, although it was not very definite, there was some support for Wolf's contention that when uncoagulated egg albumin is fed the sulphur is at first not so rapidly excreted as the nitrogen, but that later, when the excretion does start, it proceeds at a faster rate than that of the nitrogen. Wolf believed that the sulphur complex in the uncoagulated egg albumin has some power of withstanding the onset of the ferments.

Plasmon.

Only one experiment was carried out with this substance in which it was superimposed on a bread, butter and milk diet. After twelve predays of feeding, 5·9 grams of nitrogen in the form of plasmon were superimposed daily for eight days with the result that there was a consistent retention of nitrogen throughout. The mean daily output of nitrogen before the plasmon was added was 15·6 grams in the urine and faeces and despite the daily extra intake of 5·9 grams during the plasmon period the mean daily total output was only about 17·4 grams, giving a retention of nitrogen during the whole plasmon period of about 33 grams nitrogen. The four days, which made up the after-period, did not show very conspicuously the slow fall, which was to

be expected from previous work, in the output of nitrogen to the original level. It may be noted that although the subject was in very good condition throughout this experiment there was a steady although slight gain in body weight. The sulphur output showed nothing abnormal. Wolf in his plasmon experiment also found that there was no definite evidence in this case that the sulphur output preceded the output of nitrogen.

TABLE VII.

Veal feeding (bread and butter diet).

Date and hour	Total nitrogen in grms.	Total sulphur in grms.	N : S ratio	Creatinine in grms.	Weight, kilos.	Remarks
Feb. 19, 1912	9·76	—	—	—	72·3	
„ 20, „	8·62	·625	13·8	1·89	72·4	
„ 21, „	7·90	·662	11·9	1·96	72·5	
„ 22, „	7·01	·616	11·4	1·96	72·5	
„ 23, „	7·73	·664	11·6	1·93	72·6	
„ 24, „ 8–10	·575	·0436	13·2	·162	72·8	
10–12	·731	·0586	12·5	·178	—	
12– 2	·582	·0477	13·0	·172	—	
2– 4	·605	·0452	13·4	·176	—	
4– 6	·583	·0460	12·7	·178	—	
6– 8	·732	·0662	11·1	·176	—	
8–10	·680	·0663	10·3	·180	—	
10– 8	2·910	·2583	11·3	·779	—	
	7·40	·629	11·7*	1·99		
Feb. 25, 1912, 8–10	·579	·0482	12·0	·175	73·0	226 grms. stewed veal
10–12	·847	·0592	14·3	·206	—	(=10·6 grms. N) superimposed at 8.30 a.m.
12– 2	·991	·0574	17·3	·206†	—	
2– 4	·964	·0614	15·7	·196	—	
4– 6	1·131	·0773	14·6	·200	—	† Trace of creatine present.
6– 8	1·360	·0945	14·4	·191	—	
8–10	·983	·0801	12·3	·179	—	
10– 8	3·577	·3380	10·9	·750	—	
	10·43	·8081	12·9*	2·10		
Feb. 26, 1912, 8–10	·882	·0710	12·4	·179	73·0	
10–12	·929	·0702	13·2	·184	—	
12– 2	·838	·0611	13·7	·180	—	
2– 4	·778	·0616	12·6	·181	—	
4– 6	·692	·0583	11·9	·177	—	
6– 8	·809	·0710	11·4	·180	—	
8–10	·723	·0625	11·4	·182	—	
10– 8	2·932	·2658	11·0	·792	—	
	8·58	·7215	11·9*	2·05		
Feb. 27, 1912	8·16	·668	12·2	1·96	72·7	
„ 28, „	7·87	·630	12·5	1·92	72·6	
„ 29, „	7·34	·596	12·3	1·90	72·6	

* Ratios of totals.

Veal.

In this experiment 226 grams of veal containing 10·6 grams of nitrogen and ·664 gram of sulphur with a S : N ratio of 1 : 16 were superimposed on a bread and butter diet which was taken for six days previous to the day of feeding. The sixth preday urine was collected two-hourly. Following the day of feeding there were four postdays. Table VII (p. 13) gives the general result.

Here it will be noted that there is a well-marked rise in the excretion of nitrogen as the result of the feeding, an excretion which is spread over the day of feeding and the three following days. In the case of the sulphur there is also a rise in the output but the normal is regained at the end of the third day after feeding. When the percentage amounts of the intake of both nitrogen and sulphur are compared it is found that here there is no, or perhaps only a very slight, acceleration in the output of the sulphur as is seen from the following table.

TABLE VIII.

	N	S
	Percentage output	
Day of feeding	28·6	27·0
First day after	11·2	14·0
Second ,,	7·2	5·0
Third ,,	4·4	—

Thus of the nitrogen and sulphur taken in, 56·1% (including 4·7% in the faeces) of the nitrogen is excreted in four days, and 46% of the sulphur in three days.

This result fully substantiates the result obtained by Wolf who found that about 45% of the nitrogen taken in the form of veal was re-excreted within two days. He also found that the nitrogen and sulphur output ran nearly parallel, although the output curve of the sulphur was a little steeper than that of the nitrogen, indicating that the former output was slightly more rapid. It does not however agree with the results obtained by Hämäläinen and Helme, who did not find any marked retention of the nitrogen and who found a much more rapid excretion of the sulphur than of the nitrogen.

Urea.

Finally a test with urea was carried out on a comparatively low protein diet to see if, when a nitrogenous material was used which we presumed could not take the place of protein, retention of nitrogen took place. After

giving a potato and butter diet for four days twelve grams of pure urea containing 5·6 grams of nitrogen were superimposed on the breakfast meal. We found that over 95% was excreted during the day of feeding and on the subsequent day. There was thus practically no evidence of retention. Wolf obtained a similar result: he found an excretion of nearly 97% in the first two days. This result does not favour the idea that urea can replace to a certain extent the protein of the diet. If this were possible it would have been expected that under the particularly favourable conditions of our experiment, urea given on a comparatively low protein diet, a certain amount of retention of nitrogen would have occurred. The ingestion of the urea had no influence on the output of sulphur.

Creatinine Output.

The opportunity was taken in the course of the above experiments to follow the output of creatinine as it was thought that the effect of the addition of a nitrogenous substance free from creatine and creatinine to a diet poor in protein might throw some additional light on the course of the metabolism of creatine and creatinine. Our results show that the output of creatinine was scarcely affected by such additions, except of course in the case of the veal which contained creatine in small amount. This substantiates the statement of Folin that the output of the creatinine is maintained practically at a constant level.

Discussion of Results.

From these experiments then, and from others carried out by Wolf, etc., it may be concluded that Falta is right in his contention that the breakdown of protein takes place in a more or less step-like fashion. The fact that in nearly every instance there is an attempt by the cells of the organism to retain a certain amount of the nitrogen and to get rid at the same time of the excess of sulphur points to the fact that the form in which the nitrogen is retained, and apparently it is retained, although perhaps to a very limited extent, is a special one. The evidence for the storage of protein in the body is very scanty and has previously been discussed by one of us [E. P. C., 1912] at some length. Briefly it may be said that the general opinion is that a certain amount of retention can take place although the form in which the material is retained is still a matter of dispute. Some of the most interesting work in this connection is that of Müller [1907] who fed

dogs on a definite low protein diet and then amputated a limb. Subsequently the animal was fed on a high protein diet and killed after a long period of feeding. An analysis of the tissues obtained from the amputated limb on the low protein diet compared with the analysis carried out after the high protein diet showed that a certain amount of retention had taken place. Recently Diesselhorst [1911] repeated this work of Müller under practically the same conditions but making more elaborate analyses. He also found that there was a certain gain in the amount of nitrogenous material after feeding on the protein-rich diet. Grund [1910] also investigated the alterations in the composition of the tissues in starvation and after feeding. He discusses this question of the retention of reserve protein in the cells and comes to the conclusion that there is a certain amount of evidence in its favour, although he at the same time maintains that if this retention does take place it cannot play a very important part in the total metabolism. He further holds that there is a general tendency both in periods of starvation and of feeding for the tissues to maintain the same relative composition.

In conclusion we believe that from the study of the S : N ratios it is clearly shown that the increase in the output of nitrogen and sulphur which as a rule follows the ingestion of a protein meal is due to the catabolism of the protein actually ingested and not to the displacement of "effete" protoplasm from the tissues. It will be noted for example after the superimposition of the egg albumin with a S : N ratio of 1 : 8 (see Table VI) that the ratio obtained approximates very closely to the ratio of the ingested protein. This is particularly noticeable in the variations in the ratio in the two-hourly collection of the preday and the fed day. One of us [E. P. C., 1907] has previously shown that the S : N ratio in starvation when all the nitrogen and sulphur which is excreted in the urine must come from an endogenous source is about 1 : 15. If then the extra nitrogen and sulphur excreted after the ingestion of the egg albumin had come from the displacement of "effete" tissue protein it would have been expected that the S : N ratio would be nearer 1 : 15 than the 1 : 9·8 found.

Conclusions.

1. The sulphur-containing moiety of the protein after ingestion is, as a rule, more rapidly catabolised and the sulphur more rapidly excreted than the nitrogen.

2. When protein is superimposed on a low protein diet a retention of part of the nitrogen superimposed takes place.

3. The retained material is apparently stored in the tissues as a pabulum of uniform composition.

4. The rise in the output of nitrogen and sulphur after a protein meal is due to the catabolism of the actual material ingested.

5. The superimposition of protein has, in these experiments, no effect on the output of creatinine.

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II. THE FATE OF INDOLETHYLAMINE IN THE ORGANISM.

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(*Received November 10th, 1912.*)

It has been shown by several observers that the naturally occurring amino-acids are readily attacked by bacteria, and that among other products the corresponding amines are formed [Winterstein and Kuntz, 1909; Barger and Walpole, 1909; Ackermann, 1910; see Barger, 1911, for other references]. It appears probable that they are produced in the intestine in the course of the normal life of the organism and also in certain pathological conditions. Their fate after absorption is therefore a matter of considerable importance. The amines of this type have in most cases been submitted to physiological investigation [Dale and Dixon, 1909; Ackermann and Kutscher, 1910; Dale and Laidlaw, 1910], and in several cases they have been shown to be active substances. The method by which the body deals with such bases has not been investigated so thoroughly.

Some time ago we showed [Ewins and Laidlaw, 1910] that *p*-hydroxyphenylethylamine was largely converted into *p*-hydroxyphenylacetic acid and excreted as such; that the liver and the plain muscle of the body, with the exception of the plain muscle of the lung vessels, could effect the change: and that successive methylation of the amino-group rendered the base increasingly resistant to destruction in the body. Another example of a change of precisely the same type is met with in the fate of benzylamine, which was shown by Mosso [1890] to be almost quantitatively converted into benzoic acid and excreted as hippuric acid. We undertook the investigation of the mode of destruction of indolethylamine (*β*-indole-*pr*-3-ethylamine) in order to see whether it was dealt with in a similar manner, and whether its administration would increase the kynurenic acid output in dogs.

The indolethylamine used was made according to the synthesis devised by one of us [Ewins, 1911], which readily gives a good yield of the base.

We had already shown [Ewins and Laidlaw, 1910] that it was possible to obtain this base by the action of putrefactive bacteria on tryptophane; but the method is tedious and troublesome, and the yield very poor.

We have carried out two sets of experiments in the course of our investigation: (1) feeding experiments on dogs, and (2) the perfusion of surviving livers of rabbits and cats[1].

Perfusion Experiments.

The method of perfusion was similar to that already described in our paper dealing with the fate of *p*-hydroxyphenylethylamine [Ewins and Laidlaw, 1910]. Small quantities of indolethylamine were perfused through surviving livers for 3—4 hours. The perfusion fluid gradually acquired the property of giving a fine red colour, when it was mixed with one-third of its volume of strong hydrochloric acid, with the addition of one drop of dilute ferric chloride, and then boiled. The red colour occasionally had a faint blue component if the hydrochloric acid was in excess or if the boiling was prolonged. A cherry red colour also developed if a trace of sodium nitrite was substituted for the ferric chloride in the above tests. Amyl alcohol rapidly extracted the pigment from the solution, when a well-defined absorption band in the green was seen with a spectroscope. The red colour is brighter and purer and the absorption band more intense and better defined when sodium nitrite is used. When the chromogen of the pigment appeared to be fairly abundant in the perfusion fluid (usually about 3–4 hours) the perfusion fluid was collected, and the liver vessels washed through with salt solution. The combined washings and perfusion fluid were rendered faintly acid with acetic acid and boiled. The coagulated proteins were filtered off and the filtrate evaporated to small bulk. The concentrated perfusion fluid was made acid to Congo-red with strong hydrochloric acid and shaken out with ether, which readily and completely extracted the chromogen. The ethereal extracts were combined, washed twice with water and taken to dryness. If the residue were taken up in water and allowed to stand, a small quantity of a crystalline acid was regularly obtained, which when recrystallised from benzene melted at 163–164°. These crystals gave the colour reactions mentioned above with great intensity and readily gave a deep orange-red coloured picrate melting at 174°. These properties taken together are characteristic of indoleacetic acid.

[1] The animal experiments were performed by P. P. Laidlaw only.

Experiment. 0·25 grm. indolethylamine hydrochloride was perfused through a rabbit's liver for 2·5 hours. Perfusion fluid and washings were worked up as described above when 50 mgm. of indoleacetic acid were obtained. Yield 44 % of theoretical.

The identification was completed by comparison of colour reactions, melting point, and mixed melting point with synthetic β-indole-*pr*-3-acetic acid.

There can be no doubt then that indolethylamine is readily converted by the liver into indoleacetic acid, just as *p*-hydroxyphenylethylamine is converted into *p*-hydroxyphenylacetic acid.

Feeding Experiments.

One half to one gram of the hydrochloride of indolethylamine is well borne by a 7–8 kilo dog. Larger doses are inadmissible for our purpose since vomiting and sometimes purgation ensue.

The urine of the 36 hours following the administration did not give the colour reactions which we have always found in our perfusion experiments, but on adding to a small quantity of urine half its volume of strong hydrochloric acid and a trace of nitric acid and gently warming, a fine purple colour develops, which gradually (unless the mixture is cooled) becomes deep red and then orange and ultimately yellow. The colour reminds one of a very intense indican reaction. It is not, however, due to the development of indigo since at no stage can a blue component be extracted by chloroform.

We found that the chromogen of this colour reaction was an acid, which could be shaken out from the strongly acidified urine with ether or ethyl acetate, but the complete extraction of the chromogen by either of these solvents from acid urine was found to be almost impossible. The acid, however, was found to be much more readily extracted if its solubility in urine was depressed by saturation with ammonium sulphate. In a few experiments with rabbits' urine this preliminary saturation with ammonium sulphate was found to be of considerable assistance, since the procedure caused a large proportion of the hippuric acid to crystallise [cf. Roaf, 1908].

The ether or ethyl acetate extracts were carefully collected, and washed with ammonium sulphate, and then with a small quantity of water. The acid was then extracted from the ether by sodium carbonate solution. The alkaline extract was acidified, saturated with ammonium sulphate and shaken out with ether. The ether extracts were washed with water and taken to dryness. A thick gum resulted from which nothing would crystallise. The gum was

extracted with boiling water, in which most of it dissolved, excess of picric acid was added and the whole boiled with a small quantity of charcoal for five minutes. On filtration and prolonged standing the filtrate gradually deposited a deep orange-red coloured picrate. This picrate was recrystallised from water or water with a trace of acetone several times, when it separated in large, orange-red, rhomboidal plates melting at 145°. The crystals gave the colour reaction with great intensity. It appeared to us to be very probable that this acid would prove to be an indoleacetic-glycine complex, comparable with hippuric and phenaceturic acids, but for some time we were unable to obtain sufficient material to prove or disprove our assumption. The yield of picrate of the acid is not good and, as we have pointed out, the feeding experiments are limited to the administration of small quantities.

In one experiment for example, from 0·5 grm. of the hydrochloride of the base we only obtained 0·2 grm. of the picrate or a little over 20 % of the theoretical maximum yield. In some other experiments the yield was rather better but never more than 30 %.

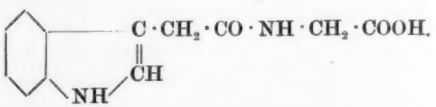
From a series of three experiments we obtained sufficient of the picrate which, although not quite pure, could be analysed:

0·0986 grams picrate gave 0·1668 CO_2 and 0·0322 H_2O

$C = 46{\cdot}1 \qquad H = 3{\cdot}6$

$C_{12}H_{12}O_3N_2 . C_6H_3O_7N_3$ requires $C = 46{\cdot}8$, $H = 3{\cdot}2$ per cent.

The figures obtained although not accurate were sufficiently so to indicate that the acidic chromogen was an indoleacetic-acid-glycine condensation product, and that the substance in question has the probable constitution denoted by the formula



For this acid we suggest the name *indoleaceturic acid*, in conformity with the nomenclature of this class of bodies to which hippuric and phenaceturic acids belong.

It may also be mentioned that quite recently Ackermann [1912] has shown that among the products of metabolism of nicotinic acid fed to a dog is found a similar glycine derivative, to which he gives the name nicotinuric acid.

Our supposition with regard to the constitution of the urinary chromogen was further confirmed by hydrolysis of the picrate with sodium carbonate.

Experiment. 0·250 grm. of the picrate was dissolved in 80 c.c. of 5 % sodium carbonate solution and hydrolysed on the water bath for one hour. On cooling and acidifying, 0·19 grm. of indoleacetic acid picrate separated out, and from the mother liquor 0·017 grm. more was obtained crystalline. Total 0·207 grm. or 95 % of the theoretical amount if the original picrate had been the suggested indoleacetic-glycine complex.

Since the amount of acid obtainable in the experiments with indolethylamine was so very small, and we have shown by perfusion experiments that indoleacetic acid is probably an intermediate step in the production of indoleaceturic acid from indolethylamine, we carried out a series of feeding experiments with indoleacetic acid in the hope of obtaining larger quantities of the desired acid. Several grams of indoleacetic acid were synthesised by a method which will be published at a later date, and given by mouth to a dog, when a 60 % yield of picrate was obtained, identical in all respects with that obtained from the feeding experiments with the base. This fact enabled us to obtain the picrate in comparatively large quantities, since the indoleacetic acid caused no symptoms. In this way we obtained without trouble 3 grams of indoleaceturic acid picrate from 3 grams of indoleacetic acid. The preparation of the free acid from the picrate could not be carried out in the usual manner (removal of the picric acid from the acidified solution by means of ether) owing to the well-marked acidic properties of the indoleaceturic acid and its solubility in ether. This difficulty was overcome by the use of the base known as nitron (1.4-diphenyl-1.3.5-endanilino-dihydrotriazole) [Busch, 1905] which precipitates picric (as well as nitric) acid as an almost insoluble salt of the base. The experiment was carried out as follows.

1·5 grams of picrate were dissolved in 150 c.c. of hot water and 1 molecular proportion of nitron dissolved in hot dilute alcohol was added. The mixture was thoroughly cooled and the precipitated picrate sucked off at the pump. The nearly colourless filtrate was neutralised with sodium carbonate, two or three drops of acetic acid added and the solution evaporated to dryness. The residue was dissolved in a few (7–8) c.c. of water and made just acid to Congo-red with dilute hydrochloric acid. An oily product separated which soon crystallised in bunches of needle-like prisms. This was filtered off, dried in vacuo over sulphuric acid for a short time and weighed. In this way 0·6 gram of the crystalline acid was obtained. This was twice recrystallised from water and then melted at 94°. The acid so obtained is very slightly soluble in cold water (about 0·2 % at ordinary temperature), almost insoluble in ligroin and benzene, easily soluble in alcohol, ether and ethyl acetate. It

crystallises from water with one molecule of water of crystallisation which is only very slowly removed by sulphuric acid in vacuo at 37°.

0·1022 gram acid (m.p. 94°) gave 0·2184 CO_2 and 0·0514 H_2O

C = 58·2 H = 5·6

Calc. $C_{12}H_{12}O_3N_2 \cdot H_2O$ C = 57·6 H = 5·6 per cent.

0·1026 gram acid (anhydrous) gave 0·2330 CO_2 and 0·0492 H_2O

C = 61·9 H = 5·3

Calc. $C_{12}H_{12}O_3N_2$ C = 62·0 H = 5·2 per cent.

This result fully confirms our supposition that the acid in question is indoleaceturic acid.

It will be observed that we have never obtained anything like a quantitative yield of indoleaceturic acid by giving indolethylamine by mouth. In our best experiments about 30 % of the base is accounted for in this manner. It is true that this probably represents a much larger quantity of indoleacetic acid, which must be the intermediate product; because the administration of indoleacetic acid has never in our experience given a quantitative yield of indoleaceturic acid. However, making some allowance for this and a little for the imperfect methods used in the isolation of the acids from such a complex mixture as urine, we still have not accounted for the whole of the base.

It appeared to us to be possible that some of the indolethylamine or indoleacetic acid might be converted into kynurenic acid, although none of the specimens of urine that we had examined showed obvious excess of this metabolite. Some experiments were therefore undertaken in which kynurenic acid estimations were made before and after administration of indolethylamine and indoleacetic acid.

A dog was kept on a standard diet of milk and dog biscuit. On this particular diet the kynurenic acid excretion was minimal, and traces only could be detected in 24 hours. The kynurenic acid was estimated by Capaldi's method [1897].

Experiment.

Date	Volume of urine	Kynurenic acid	Remarks
Sept. 23—24	400 c.c.	Trace	—
Sept. 24—25	440 c.c.	Trace	—
Sept. 25—26	600 c.c.	·480 grm.	On morning of 25th 1·6 grm. tryptophane by mouth.
Sept. 30—Oct. 1	350 c.c.	Nil	—
Oct. 1—2	600 c.c.	Trace	On Oct. 1st 1·6 grm. indoleacetic acid by mouth.
Oct. 2—4	850 c.c.	Traces	—
Oct. 4—5	470 c.c.	Traces	On Oct. 4th 1·0 grm. indolethylamine by mouth.

Neither indolethylamine nor indoleacetic acid increases the kynurenic acid output in dogs.

It seems clear that this path of metabolism of indole-derivatives does not aid us in accounting for the quantitative difference between the ingested substances and those excreted.

Samples of urine were examined in various ways for other possible end products but without success, and we suggest that the portion of the base unaccounted for is completely broken up in the body.

Occurrence of Indoleaceturic Acid.

We have some evidence of the occurrence of indoleaceturic acid in normal urine of herbivora. We have found that an acidic, ether-soluble substance, giving the purple colour reaction, occurs in small quantity in the urine of rabbits, but attempts at isolation of the acid or its picrate have always failed. The small amount of the chromogen cannot be separated from the other acid, ether-soluble substances present in all urines.

In examining urine for indican it occasionally happens that a fine purple colour is produced by hydrochloric acid and an oxidising agent but no blue component can be extracted by chloroform. Here, again, it is suggested that the chromogen is indoleaceturic acid, but considerable quantities of urine would be required to isolate the acid and such have not been at our disposal.

Herter [1908] described a case of a young girl who was suffering from some unusual intestinal infection and from whose faeces an unusual organism was isolated. The urine of the patient gave a marked urorosein reaction if the urine were stale. Indoleacetic acid was isolated from this urine and Herter identified it as the chromogen of urorosein originally described by Nencki and Sieber in 1882. The colour, solubilities and other characteristics of the pigment agree very well with those which Nencki and Sieber described as characteristic for urorosein, although other observers have described other substances as the chromogen of the same pigment. It is unfortunate that Herter gives no description of the method of isolation of indoleacetic acid from the urine of his patient. It is quite possible that it was originally present as indoleaceturic acid and that this was hydrolysed to indoleacetic acid in the process of isolation, for the complex acid is readily hydrolysed by weak alkalies such as sodium carbonate. The fact that stale urine gave a colour, while fresh did not, was attributed by Herter to the formation of nitrites. It is quite possible that the indoleaceturic acid was decomposed into indoleacetic acid and glycine, just as hippuric acid is readily split up by bacteria, and

that the nitrites, though certainly playing a part, did not furnish the whole explanation.

If it could be demonstrated that indoleaceturic acid was a normal constituent of urine it would by no means follow that its precursor in the body was indolethylamine, for bacteria often produce indoleacetic acid from tryptophane and proteins; in fact it is a more frequent product of bacterial action than indolethylamine.

SUMMARY.

1. Indolethylamine is converted, by the perfused liver, into indoleacetic acid.

2. Indoleacetic acid is excreted in combination with glycine forming indoleaceturic acid.

3. About 30% of a given dose of indolethylamine is excreted as indoleaceturic acid in dogs.

4. Neither indolethylamine nor indoleacetic acid affects the kynurenic acid output in dogs.

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III. THE HYDROLYSIS OF GLYCOGEN BY DIASTATIC ENZYMES. COMPARISON OF PREPARATIONS OF GLYCOGEN FROM DIFFERENT SOURCES.

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(*Received November 23rd 1912.*)

For some time considerable uncertainty has prevailed as to whether samples of glycogen prepared from different sources are identical, the evidence on this point being in many respects contradictory. It is agreed that all have the same empirical formula $C_6H_{10}O_5$, the different results obtained by some observers being undoubtedly due to insufficient drying of their glycogen preparations. For Harden and Young [1902] found that the last traces of water can only be removed by heating to 100°C. *in vacuo* over phosphorus pentoxide, a procedure which had not been adopted by previous workers. Very divergent values have however been obtained for the specific rotation of different preparations of glycogen, the results covering a range of about 30°. Thus Cremer [1894] found as the $[\alpha]_D$ of yeast glycogen + 198·9°, while Clautriau's [1895] result was 184·5°. Harden and Young [1902] also obtained for oyster and rabbit glycogens the value 191·2° while with yeast glycogen the mean of several determinations was 198·3°, a result in close agreement with that found by Cremer. It is uncertain however whether these differences are of any real significance, the strong opalescence of glycogen solutions making necessary the use of very dilute solutions and thereby introducing a large experimental error.

Other points of difference have however been noticed by various observers, though again the results are contradictory. Cremer for example states that yeast glycogen gives a darker colouration with iodine than does oyster glycogen, while other workers have obtained exactly opposite results. In a similar way different preparations of glycogen yield solutions with varying degrees of opalescence but these variations are by no means constant either in nature or extent. At present it is impossible to explain these results and

at the same time it is uncertain how far divergences of this nature imply any real differences between the respective glycogens.

A more valuable comparison was made by Harden and Young when they measured the rate of hydrolysis of different glycogen solutions by dilute acids. The course of hydrolysis was followed by estimations of the polarimetric and reducing powers of the solutions. By this method they were unable to detect any differences between rabbit, yeast and oyster glycogens and came to the conclusion that they were in all probability identical.

It seemed possible however that a study of the behaviour of various preparations of glycogen towards diastatic ferments might prove a more delicate means of detecting any difference in their constitution, for it has on many occasions been pointed out what marked influences even small alterations in the molecule may exert on the course of enzyme action. It is the results of such an investigation which are presented in this communication. Four preparations of glycogen have been employed, these being derived from (*a*) liver of dog, (*b*) liver of rabbit, (*c*) oyster and (*d*) yeast. The enzyme used was an extract of pig's pancreas, this being one of the most convenient sources for obtaining active preparations. Before making any direct comparison, the general conditions of action were studied, for although a great amount of work has been carried out on the behaviour of starch towards diastatic enzymes, the hydrolysis of glycogen has received very much less attention. These experiments will be first described.

Preparation of Glycogen.

A. Dog. A large dog was fed with considerable quantities of carbohydrate for forty-eight hours and then killed. The liver was rapidly removed, minced and heated on the water bath with 60 per cent. caustic potash. The glycogen was precipitated from this solution in the usual way [Pflüger, 1905] and purified by repeated reprecipitation from its aqueous solution, the first two or three precipitations being carried out with solutions rendered faintly acid with acetic acid. Glycogen was in this way obtained in considerable quantity, free from nitrogen and containing only a trace of ash. In the final stages it is frequently difficult to produce a satisfactory precipitation with alcohol alone, the addition of a small quantity of acetone in these cases, however, readily brings down all the glycogen, leaving a liquid which filters well.

B. Rabbit. The method was the same as that described above, a dozen rabbits being used for each preparation. They were previously fed on large quantities of carrot.

C. Oyster. A gross of oysters (1500 grms.) were worked up by Pflüger's method and yielded 120 grms. purified glycogen.

D. Yeast. The glycogen was separated and purified from yeast gum by the method recently described by Harden and Young [1912] with the exception that yeast juice was employed as the starting material. Large quantities of yeast juice were boiled, filtered and the filtrate precipitated with alcohol. The precipitate was then heated on the boiling water bath with 60 per cent. caustic potash to remove protein, the solution diluted and again precipitated with alcohol. After thorough washing with 50 per cent. alcohol and reprecipitation, the glycogen was purified from yeast gum by saturation of the solution with ammonium sulphate, which precipitates the glycogen but not the gum. This process was repeated three times and the glycogen then dialysed to remove the ammonium sulphate. The solution was then again precipitated with alcohol and the further purification carried out as in the final stages described above. The preparation is extremely tedious and the yield very variable according to the history of the yeast employed.

The ash content of the four specimens was as follows. Dog glycogen, 0·38 per cent. Oyster, 0·2 per cent. Rabbit, 0·51 per cent. Yeast, 0·87 per cent. The reaction of the two latter was faintly alkaline, while the dog and oyster preparations both yielded slightly acid solutions.

Preparation of Enzyme.

100 grms. of pig's pancreas were minced and well ground up with half the weight of sand. Sufficient kieselguhr was added to form a fairly stiff mass which was then pressed out in a hydraulic press. The extract obtained in this way after filtration yielded a clear yellow-brown liquid which was extremely active. It was usually diluted to five or ten times its volume before use.

Experimental methods.

The course of the hydrolysis can be followed in several ways: (i) estimations of the residual glycogen, (ii) determinations of the optical activity, and (iii) estimations of the reducing power of the solution after known intervals of time. The first of these methods however is unsatisfactory owing to the fact that some of the higher dextrins are also precipitated by alcohol as well as the glycogen, for a precipitate is still obtained in this way some time after the disappearance of the iodine reaction. The optical activity of the solution also is due to a complex mixture of substances, the nature of which has not been thoroughly worked out and hence in the majority of the following experiments, the hydrolysis has been

followed by estimations of the reducing power alone, though this method is not altogether free from the same objection. The latter were carried out by Bertrand's method and the reducing power calculated as maltose, preliminary experiments having shewn that under the conditions employed very little if any hydrolysis of the maltose into glucose took place. The reducing power of a maltose solution of known strength was first determined and from the results obtained, a curve constructed from which the maltose corresponding to any found weight of copper could be read off.

The experiments were all carried out in flasks of Jena glass which were used for no other purposes. In most cases toluene was added to the solutions to prevent bacterial contamination.

Hydrolysis of Glycogen by an Extract of Pig's Pancreas.

To study the general course of the hydrolysis, 500 c.c. of a 1 per cent. solution of oyster glycogen were incubated with 2·5 c.c. of a pancreas extract with the addition of 5 c.c. toluene as antiseptic. The glycogen and enzyme solutions were each brought to the temperature of the bath (37°) before mixing and then after known intervals of time, samples of the liquid were removed and the following estimations made.

A. Reducing power.

B. Matter precipitable by alcohol (glycogen and higher dextrins). The precipitation was carried out by adding two volumes of alcohol. At the same time the reaction of the solution towards iodine was observed.

The results are collected in the following table.

TABLE I.

Hydrolysis of Glycogen by Pancreatic Diastase.

Time	Duration of action	Wt. of ppt. thrown down by 2 vols. alcohol	Maltose	Iodine reaction	Percentage hydrolysis
12.30	0	4·702 grms.	—	Dark red brown	0
12.35	5 mins.	—	0·223 grm.	Red brown	4·45
12.45	15 ,,	3·49 ,,	0·664 ,,	,,	13·32
1.00	30 ,,	2·74 ,,	1·156 grms.	,,	23·2
1.15	45 ,,	2·30 ,,	1·471 ,,	Pale red brown	29·5
1.30	60 ,,	1·83 ,,	1·714 ,,	Very faint	34·4
2.0	90 ,,	1·48 ,,	2·003 ,,	None	40·2
2.30	2 hrs.	1·20 ,,	2·145 ,,	—	43·0
3.30	3 ,,	—	2·292 ,,	—	46·0
4.30	4 ,,	0·65 grm.	2·362 ,,	—	47·4
5.30	5 ,,	—	2·434 ,,	—	48·8
9.30	9 ,,	Estimation spoilt	2·550 ,,	—	51·2

In Fig. 1 the results are shewn graphically, curve A representing the degree of hydrolysis and curve B the residual glycogen and dextrins precipitable by two volumes of alcohol. It will be seen that the action commences at a high initial velocity and proceeds for a very brief period in a linear direction. By the time the iodine reaction has disappeared, the rate has slowed down considerably and finally a long period of extremely slow hydrolysis follows. It would appear that the rapid initial stage is chiefly concerned with the conversion of the glycogen into dextrins with simultaneous

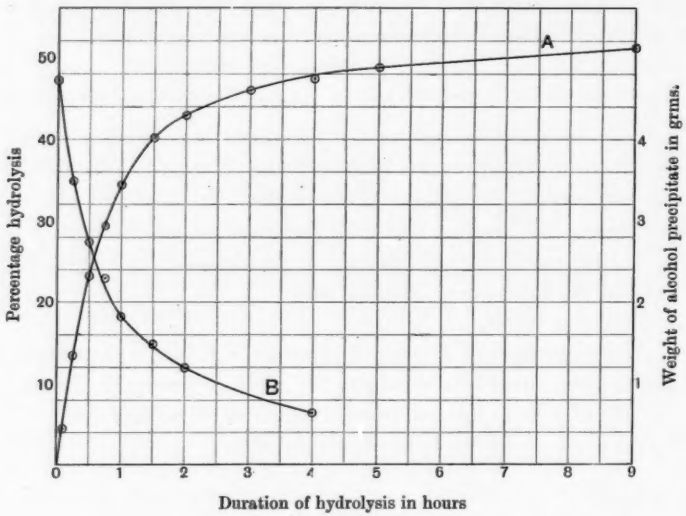

Fig. 1.

production of sugar. These dextrins, which are very resistant to further hydrolysis, are then slowly broken down to sugar. It is obvious however that in the above experiment this second stage would never have reached completion and this seems to be the general rule unless very high enzyme concentrations are employed. The iodine reaction disappeared when about 40 per cent. of the glycogen had been completely hydrolysed to maltose. The action in short closely resembles the hydrolysis of starch but is slower and less complete.

Influence of Temperature.

A series of flasks each containing 50 c.c. of 1 per cent. glycogen were incubated with 0·5 c.c. enzyme solution, at temperatures varying from 25° to 50°. Thirty minutes after the addition of the enzyme, the action was stopped by adding 5 c.c. of N. KOH, the liquid was at once cooled and the volume made up to 100 c.c. The reducing power of 20 c.c. of this dilution was then estimated. The results are shewn below in Table II and graphically in Fig. 2. For the sake of comparison the results of a starch hydrolysis with the same enzyme preparation are given also.

TABLE II.

Influence of Temperature.

	A. Glycogen		B. Starch	
Temperature of incubation	Total maltose	Percentage hydrolysis	Total maltose	Percentage hydrolysis
25·5°	0·1070	20·2	—	—
27·5°	—	—	0·1310	24·7
34°	0·1212	22·9	—	—
37°	0·1375	25·9	0·1545	29·2
39·5°	0·1330	25·1	—	—
42°	0·1310	24·7	0·1700	32·1
46°	0·1260	23·7	0·1895	35·7
50·5°	0·1190	22·5	0·1760	33·2

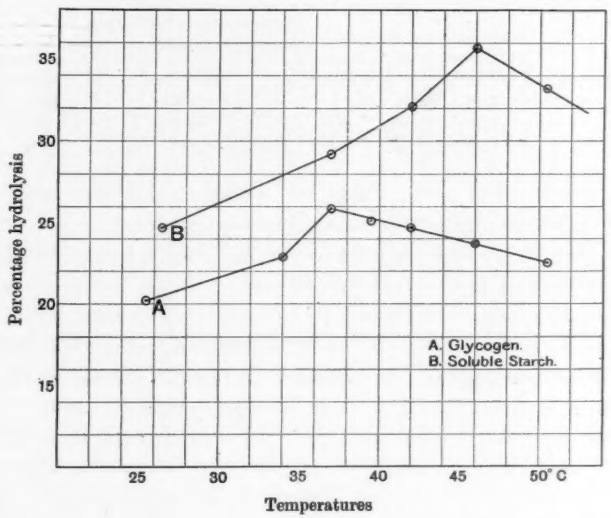

Fig. 2.

Hence with the enzyme employed in these experiments, the optimum temperature for glycogen hydrolysis was about 37°C. and this value was unchanged by substituting different preparations of glycogen. For starch hydrolysis on the other hand the optimum temperature found with the same enzyme solution was 46°C. This result therefore lends some support to the hypothesis that the two actions are brought about by two distinct enzymes.

Influence of Enzyme Concentration.

Three dilutions of the enzyme solution were prepared and 0·5 c.c. of each added to a series of flasks each containing 50 c.c. of a 1 per cent. glycogen solution (oyster) + 1 c.c. toluene and previously brought up to 37°. Altogether three flasks were used with each dilution.

After 5, 15, and 25 minutes' incubation one flask of each series was removed from the bath and the action stopped by the addition of 5 c.c. N. alkali. The reducing power of each solution was then determined and the results are shewn in Table III below.

TABLE III.

Influence of Enzyme Concentration.

Each flask contained 50 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

Exp.	Dilution of enzyme solution	Reduction after time given, mgrms. Cu from 20 c.c. liquid		
		5 mins.	15 mins.	25 mins.
A	1/10	7	21·5	32·0
B	1/20	3·35	10·0	—
C	1/40	1·7	5·2	9·0

These results shew very clearly two things. (i) Under the above conditions the rate of hydrolysis is directly proportional to the concentration of enzyme, and (ii) the action is a linear one, the reduction after 15 minutes' being almost exactly three times that after five minutes' incubation and so on. These results only obtain however when very dilute enzyme preparations are used and during the initial part of the experiment—in other words while there is a considerable excess of unchanged glycogen. Similar results have been obtained by Evans [1912] in the hydrolysis of starch by saliva.

When stronger preparations of enzyme are employed the above relations can only be observed for a very brief period of the hydrolysis, but another point now becomes more noticeable. With dilute enzyme preparations, the action comes to an end long before all the glycogen has been completely

converted into maltose. This is apparent in the results quoted in Table I where it will be noticed that after nine hours there was only a 51 per cent. hydrolysis and the action had practically come to an end. When however higher concentrations of enzyme are employed, the end point of the reaction is moved very much nearer to complete hydrolysis as will be seen from the experiments described below.

A series of flasks was incubated at 37°C. containing the following solutions:

A. 100 c.c. 1 per cent. glycogen solution + 4·9 c.c. H_2O + 0·1 c.c. enzyme + 1 c.c. toluene.

B. 100 c.c. 1 per cent. glycogen solution + 4·5 c.c. H_2O + 0·5 c.c. enzyme + 1 c.c. toluene.

C. 100 c.c. 1 per cent. glycogen solution + 4·0 c.c. H_2O + 1·0 c.c. enzyme + 1 c.c. toluene.

D. 100 c.c. 1 per cent. glycogen solution + 0 c.c. H_2O + 5·0 c.c. enzyme + 1 c.c. toluene.

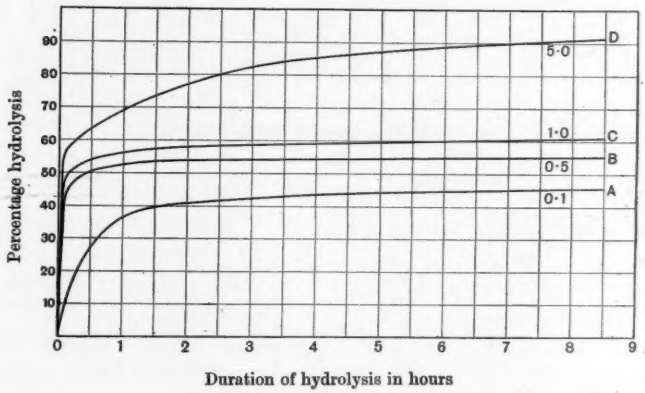

Fig. 3.

The total volume in each case was the same, the only difference being the concentration of enzyme. After 8½ hours the action was almost over in all four solutions and the amount of hydrolysis which had taken place was as follows:

A, 45·6 per cent. B, 55·1 per cent. C, 60·7 per cent. D, 91·3 per cent. The complete course of the hydrolysis in each case is shewn in Fig. 3.

Very similar results were obtained by Philoche [1908] with taka diastase and it is not quite clear what interpretation is to be placed upon them, for, as

will be seen, there appears to be no direct relationship between the concentration of enzyme and the final degree of hydrolysis. The influence of the products formed during the hydrolysis may be partially responsible but judging from experiments described later, it is unlikely that this is the sole cause. The incompleteness of the hydrolysis cannot be ascribed to the destruction of the enzyme, for the latter after 20 hours' incubation rapidly attacked a second quantity of glycogen.

Influence of Glycogen Concentration.

Unless very low concentrations are used—less than 0·5 per cent. with the strength of enzyme I have generally employed—the *initial* rate of hydrolysis is practically unchanged by increasing the glycogen concentration. In the weaker solution, however, the rate naturally begins to fall at an earlier period owing to the glycogen being more rapidly exhausted. These points are illustrated by the results shewn in Table IV.

TABLE IV.

Influence of Glycogen concentration.

Time after addition of enzyme	Reduction, mgrms. Cu from 20 c.c. liquid		
	A. 1% glycogen	B. 2% glycogen	
5 mins.	9·0	9·0	Rates equal.
15 ,,	26·8	27·0	
30 ,,	45	52	Rate in A falling, B practically unchanged.
1 hr.	66	89	Both rates falling, but more rapidly in A.
4 hrs.	92	147	
20 ,,	108	188	Action at an end in both.
% hydrolysis	49·6	43·5	

Influence of Products.

As previously stated, unless high concentrations of enzyme are employed the hydrolysis is not complete. In order to see whether this was due to the influence of the products the following experiments were carried out.

Influence of Maltose. In a preliminary experiment the action had stopped when about 50 per cent. of the glycogen had been hydrolysed to maltose, i.e. when the solution contained rather over 0·5 per cent. of maltose. This

amount of maltose was therefore added to one flask at the beginning of the experiment and the rate of hydrolysis compared with one containing no sugar at the beginning. The composition of the two solutions was as follows:

Flask A. 100 c.c. 1 per cent. glycogen + 1 c.c. toluene + 0·5 c.c. enzyme.

Flask B. 100 c.c. 1 per cent. glycogen solution containing 0·5 grm. maltose + 1 c.c. toluene + 0·5 c.c. enzyme.

TABLE V.

Influence of Maltose.

Time after addition of enzyme	A. Glycogen alone	B. Glycogen + maltose		
	Mgrms. Cu from 20 c.c.	Mgrms. Cu from 20 c.c.	Cu due to added maltose	Cu due to maltose formed
15 mins.	27	136	108	28
30 ,,	46	154	,,	46
1 hr.	66	173	,,	65
2 hrs.	80	188	,,	80
4 ,,	92	199	,,	91
8 ,,	100	207	,,	99
20 ,,	108	212	,,	104
% hydrolysis	49·6			47·6

It will be seen from these results that the rate of hydrolysis was practically unchanged by the maltose added and the final degree of hydrolysis lowered by only two per cent. The dextrins formed during the course of the reaction, however, seemed to have some retarding influence, this being illustrated by the following experiment.

Influence of Dextrin. 100 c.c. of 2 per cent. glycogen were incubated at 37° with 0·5 c.c. enzyme until the solution no longer gave the iodine reaction. The action was then stopped by heating the flask in a boiling water-bath. The solution thus obtained was free from glycogen and in addition to the dextrins contained about 0·9 per cent. of maltose. It is referred to below as "products of hydrolysis." The following mixtures were then made up and incubated at 37°.

A. 50 c.c. 2 per cent. glycogen + 50 c.c. "products of hydrolysis" + 1 c.c. toluene + 0·5 c.c. enzyme.

B. 50 c.c. 2 per cent. glycogen + 50 c.c. H_2O + 1 c.c. toluene + 0·5 c.c. enzyme.

Incubation was continued for six hours, the reducing power of the two solutions being estimated from time to time. The results are collected in Table VI.

TABLE VI.

Influence of Products of Hydrolysis.

Time after addition of enzyme	B. Glycogen alone	A. Glycogen + products		
	Reduction, mgrms. Cu from 20 c.c. liquid	Reduction, mgrms. Cu from 20 c.c. liquid	Cu due to sugar present at start	Cu due to maltose formed
15 mins.	26	110	84	26
30 ,,	45	128	,,	44
1 hr.	66	149	,,	65
2 hrs.	81	159	,,	75
4 ,,	90	166	,,	82
6 ,,	97	170	,,	86
% hydrolysis	44·6			39·3

In this case although there was again no difference in the initial rates, after one hour the hydrolysis in A began to lag behind that in B and after six hours only 39·3 per cent. of the glycogen in A had been hydrolysed as against 44·6 per cent. in B. Moreover in A the action had almost stopped while in B it was still proceeding at a fair rate. We have here then a marked hindering action which must apparently be due to the dextrin-like substances formed during the hydrolysis.

Influence of Acidity or Alkalinity.

The ash almost always present in samples of glycogen consists to a large extent of phosphates and according to the nature of these phosphates the solution may have either an acid or alkaline reaction. Before comparing different samples of glycogen it was therefore necessary to determine to what extent this difference in reaction would influence the rate of hydrolysis. It is obvious moreover that the optimum dose of acid will vary according to the source and proportion of the enzyme used, for the latter as well as the solution acted on may contain substances capable of neutralising some of the acid. On the other hand the optimum hydrogen ion concentration is a constant independent of these factors. In the latter case we obtain a measurement of the true optimum reaction of the solution, while in the former we have only an apparent value, the degree of accuracy of which will depend on the amount and nature of the salts present in the solution. Thus Sörensen [1909] working with three different preparations of invertin found the optimal dose of sulphuric acid (apparent acidity) to be very different for the three preparations but the optimum hydrogen ion concentration (true

acidity) was the same in each case. Hence in all the following experiments the reaction of the solution has been determined by measurements of the hydrogen ion concentration.

At first attempts were made to do this by means of the colorimetric method described by Sörensen. The strong opalescence of the glycogen solutions however rendered this method impracticable and hence the electrical method has been used throughout. The apparatus employed was that of Michaelis and Rona [1909] which is especially suitable since only small quantities of solution are required. In this method, the electrodes are kept in a still atmosphere of hydrogen and connection is made between the two cells by means of a tape soaked in a solution of potassium chloride. By making two determinations using different concentrations of KCl, e.g. 1·75 N. and 3·5 N., the contact potential due to the salt can be calculated and corrected for and, as equilibrium is established almost immediately, the method is both rapid and convenient. The determinations were all made at 37°, the temperature at which the hydrolyses were carried out, the two operations being conducted in the same thermostat. In each case the standard solution against which the glycogen solutions were compared was N/10 HCl and from the value of the E.M.F. thus set up and determined, the hydrogen ion concentration was calculated in the usual way[1].

The latter is denoted by the sign p_H, this being the logarithm of the reciprocal value of the factor of normality. p_H being known, the hydroxyl ion concentration can be calculated from the dissociation constant of water. By graphical interpolation from a series of determinations by Lundén [1907], the value at 37° has been taken as $2{\cdot}45 \times 10^{-14} = 10^{-13{\cdot}61}$. Hence in a N/100 solution of hydrogen ions where $p_H = 2$ the hydroxyl ions will have a concentration at 37° of $10^{-11{\cdot}61}$ and so on. In the same way at absolute neutrality the concentration of both ions will be $10^{-6{\cdot}8}$.

Determination of Optimum Reaction.

To a series of flasks each containing 100 c.c. of a 1 per cent. glycogen solution, were added varying amounts of N/100 NaOH or H_2SO_4. The total volume of the solutions, however, was kept the same in every case. Each flask was then in turn placed in the bath at 37° and when the solution had acquired that temperature 0·5 c.c. of a dilute enzyme preparation was added.

[1] A full account will be found in Sörensen's paper [1909] where he also discusses the possible sources of error in the method.

After thorough mixing, a sample was removed and the hydrogen ion concentration at once determined. Thirty minutes after the addition of the enzyme, a second sample was taken, the action stopped by the addition of a small quantity of N. alkali and the reducing power estimated. In this way the true reaction and the degree of hydrolysis was determined in each case. This process was repeated with all four preparations of glycogen. In these experiments no toluene or other antiseptic was used, Sörensen having found that these substances interfere somewhat in the hydrogen ion determinations, equilibrium not being so readily established. As the duration of each experiment, however, was only 30 minutes it is highly improbable that this in any way influenced the results. These are collected in Table VII and also shewn graphically in Fig. 4.

TABLE VII.

Influence of hydrogen ion concentration on the rate of hydrolysis.

Experiment	Source of glycogen	C.c. of N/100 acid or alkali added	E.M.F. volts	p_H	Reduction, mgrms. maltose in 20 c.c.	% hydrolysis in 30 mins.
1	Dog liver	3·0 H_2SO_4	0·2055	4·43	7·37	3·7
2	,,	1·0 ,,	0·2306	4·84	33·5	16·8
3	,,	0·5 ,,	0·2754	5·57	52·9	26·6
4	,,	0 ,,	0·2940	5·87	59·0	29·6
5	,,	0·25 NaOH	0·3018	6·00	61·4	30·8
6	,,	0·5 ,,	0·3217	6·32	60·3	30·3
7	,,	1·0 ,,	0·3410	6·63	54·0	27·1
8	,,	3·0 ,,	0·3755	7·19	37·1	18·6
9	,,	6·0 ,,	0·4078	7·72	12·1	6·1
14	Oyster	0·5 H_2SO_4	0·2260	4·76	28·0	14·1
15	,,	0 ,,	0·2730	5·53	40·4	20·3
16	,,	0·5 NaOH	0·2870	5·76	44·2	22·2
17	,,	1·0 ,,	0·3019	6·00	44·4	22·3
18	,,	3·0 ,,	0·3322	6·49	32·8	16·5
19	,,	6·0 ,,	0·3980	7·56	10·7	5·4
22	Rabbit liver	6·0 H_2SO_4	0·2492	5·14	4·8	2·4
23	,,	5·0 ,,	0·2750	5·56	35·6	17·9
24	,,	4·75 ,,	0·2850	5·72	45·2	22·7
25	,,	4·5 ,,	0·3010	5·97	47·5	23·9
26	,,	4 0 ,,	0·3180	6·26	47·0	23·6
27	,,	3·0 ,,	0·3412	6·64	42·3	21·3
28	,,	0 ,,	0·3818	7·30	24·9	12·5
29	Yeast	6·0 H_2SO_4	0·2748	5·55	25·5	12·8
30	,,	5·0 ,,	0·2940	5·87	39·5	19·8
31	,,	3·0 ,,	0·3120	6·16	40·0	20·1
32	,,	1·5 ,,	0·3245	6·36	39·5	19·8
33	,,	0 ,,	0·3580	6·91	27·1	13·6

The results shewn above indicate that the value obtained for the optimum hydrogen ion concentration was practically the same with each preparation of glycogen, that from oyster however giving a slightly lower figure than the other three. The optimum reaction therefore for the enzyme I have used was just on the acid side of the neutral point, the hydrogen ion concentration corresponding to an acidity of about $N/10^6$. Alteration of the reaction in either direction produced a considerable reduction in the rate of hydrolysis especially in the case of the dog and oyster glycogens. Hence in comparing the rate of hydrolysis of different glycogen preparations it is essential to see that the reactions of the solutions are as nearly the same as possible, if the results are to be at all reliable.

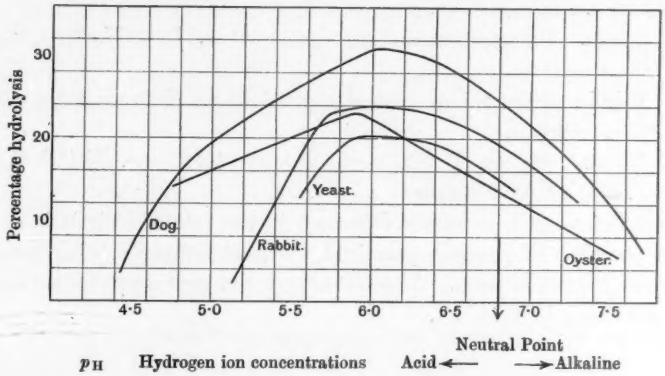

Fig. 4.

Comparison of Glycogens.

1 per cent. solutions of the four kinds of glycogen were prepared and sufficient N/100 acid or alkali added so that each solution after the addition of the enzyme was as nearly as possible at the optimum reaction. The amount to be added was estimated from the results obtained in the experiments last described, the total volume was the same in each case. Immediately after the addition of the enzyme, the hydrogen ion concentration was determined to make sure that the reaction was really correct and then after 30 minutes' hydrolysis, the action was stopped by the addition of alkali and the reducing power estimated. Both the hydrolysis and the hydrogen ion determination were carried out at 37° and no toluene was used for the reason already stated. The results are shewn in Table VIII.

TABLE VIII.

Comparison of Glycogens. I.

Source of glycogen	E.M.F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis after 30 mins.	Relative rates
Dog	0·2970 volts	5·92	46·10	23·05	100
Rabbit	0·2986 ,,	5·94	43·25	21·62	93·82
Oyster	0·2956 ,,	5·90	41·0	20·5	88·9
Yeast	0·3014 ,,	5·99	39·5	19·7	85·7

COMPOSITION OF SOLUTIONS.

A. Dog glycogen. 100 c.c. 1 per cent. glycogen + 0·25 c.c. N/100 NaOH + 4·5 c.c. H_2O + 0·5 c.c. enzyme.

B. Oyster. 100 c.c. 1 per cent. glycogen + 1·0 c.c. N/100 NaOH + 4·0 c.c. H_2O + 0·5 c.c. enzyme.

C. Rabbit. 100 c.c. 1 per cent. glycogen + 4·5 c.c. N/100 H_2SO_4 + 0·5 c.c. H_2O + 0·5 c.c. enzyme.

D. Yeast. 100 c.c. 1 per cent. glycogen + 4·25 c.c. N/100 H_2SO_4 + 0·75 c.c. H_2O + 0·5 c.c. enzyme.

There is thus quite a marked difference in the rate of hydrolysis of the four preparations of glycogen examined, the least difference being between those from oyster and yeast. A second experiment gave very similar results which are shewn in Table IX.

TABLE IX.

Comparison of Glycogens. II.

Source of glycogen	E.M.F. of solution against N/10 HCl at 37° C.	p_H	Mgrms. maltose per 20 c.c. after 30 mins.	Percentage hydrolysis	Relative rates
Dog	0·3046 volts	6·0	48·9	24·45	100
Rabbit	0·2950 ,,	5·9	46·1	23·05	94·3
Oyster	0·2901 ,,	5·8	42·4	21·2	86·7 *
Yeast	0·3112 ,,	6·1	40·45	20·2	83 †

* Solution rather too acid. † Solution rather too alkaline.

OPALESCENCE OF GLYCOGEN SOLUTIONS.

A 0·2 per cent. solution was made up of each of the four preparations of glycogen and the opalescence of these compared. This was carried out in Nessler glasses, the volume of solution being adjusted until the opalescence appeared to be the same in each case. The comparison is not easy and the experimental error is therefore large but the solution of rabbit glycogen was

markedly the most opalescent of the four examined, oyster and dog glycogen being about equal and yeast less opalescent than any. The actual results obtained were as follows:

TABLE X.

Glycogen	Volumes of 0·2 solutions having equal opalescence	Relative opalescence
Rabbit	11 c.c.	4·5
Dog	30 ,,	1·7
Oyster	33 ,,	1·5
Yeast	50 ,,	1

Colouration with Iodine.

To 10 c.c. of a 0·2 per cent. solution of each of the glycogen preparations was added 1 c.c. of a 1 per cent. solution of iodine. The volume in each case was then made up to 50 c.c. and the colours compared in Nessler glasses with the following results:

TABLE XI.

Glycogen	Volumes of solutions giving equal depth of colour	Relative strength of colouration
Rabbit	10·5 c.c.	4·7
Dog	19 ,,	2·6
Yeast	32 ,,	1·5
Oyster	50 ,,	1

Thus both as regards opalescence and the colouration with iodine there is a distinct difference between the four glycogen solutions.

Summary and Conclusions.

The conditions under which glycogen is hydrolysed by a pancreatic extract (pig) have been studied with the following results:

1. The hydrolysis at first proceeds with great rapidity, the glycogen being quickly converted into dextrins with simultaneous production of maltose. The further breaking down of these dextrins takes place with extreme slowness and is as a rule incomplete.

2. The optimum temperature for glycogen hydrolysis of the enzyme employed in these experiments was 37°. Below this there is a rapid fall in the rate, between 37° and 50° this is less marked. With starch the optimum temperature for the same enzyme preparation was 46°.

3. While excess of glycogen is present, the rate of change is directly proportional to the enzyme concentration and the action is a linear one.

4. With low enzyme concentrations, the action is by no means complete; the degree of hydrolysis, however, rises with increasing concentration of enzyme though not in the same ratio.

5. The concentration of the glycogen solution has little influence on the initial rate of hydrolysis unless very low concentrations (less than 0·5 per cent. in my experiments) are employed.

6. The action is hindered to a small extent by the products of hydrolysis, the dextrins probably being the interfering substances.

7. The action is favoured by the presence of small traces of acid, the optimum reaction determined with several preparations of glycogen being a hydrogen ion concentration of 10^{-6} (at neutrality this value is $10^{-6\cdot8}$ at 37°).

8. Samples of glycogen prepared from (i) dog, (ii) rabbit, (iii) oyster, and (iv) yeast are hydrolysed at different rates when compared at the optimum hydrogen ion concentration. Taking dog glycogen as 100, the relative rates of hydrolysis found are, rabbit 94, oyster 88, and yeast 84.

9. The degree of opalescence and the colouration with iodine exhibited by glycogen solutions vary with the source from which the glycogen has been obtained.

10. In view of the above points of difference it is probable that the four preparations of glycogen examined are not identical in constitution, though it is possible these differences might be caused by variations in the colloidal state of the four glycogen solutions. If, however, the glycogens are distinct, the diastatic enzymes obtained from different animals should also be specific and experiments are in progress to test this point.

My thanks are due to Professor Arthur Harden, F.R.S. and to Dr H. MacLean for much useful advice during the course of this research.

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IV. THE METABOLISM OF ORGANIC PHOSPHORUS COMPOUNDS. THEIR HYDROLYSIS BY THE ACTION OF ENZYMES.

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(*Received November 23, 1912.*)

Introduction.

Our knowledge concerning the composition of the organic phosphorus compounds occurring in both plants and animals is still very rudimentary. Five groups of organic phosphorus compounds occur in nature:—

1. *Phospholipines*, of which lecithin is the principal example, and in which the phosphorus is regarded as being in ester combination with glycerol as glycerophosphoric acid;

2. *Phytin*, in which phosphoric acid is in combination with inositol as inositol-phosphoric acid, the compound itself being regarded as the calcium magnesium salt;

3. *Hexosephosphate*, in which a hexose is combined with phosphoric acid;

4. *Nucleic acid*, in which again a carbohydrate (hexose or pentose) is combined with phosphoric acid, and attached to the carbohydrate is a purine base;

5. *Phosphoprotein*, in which phosphoric acid is in an unknown form of combination, presumably with one of the amino-acids composing the protein molecule.

The phospholipines occur chiefly in animals and only to a small extent in plants; phytin or phytic acid has only been found in plants; hexosephosphate is a product formed in alcoholic fermentation by yeast; nucleic acid is said to be the chief constituent of the cell nuclei in both plants and animals; phosphoprotein is present only in the animal kingdom and forms the chief protein foodstuff of embryo birds and suckling mammals.

Considering how little is known as to the chemical composition of these

compounds it is not to be wondered at that the knowledge of their metabolism is vague. Most investigators will agree that plants can synthesise organic phosphorus compounds from inorganic phosphates and that they are also able to carry out the reverse process, but how these changes are effected, or by what enzymes they are caused, is practically unknown.

Most of the experiments on phosphorus metabolism in animals have been feeding experiments in which animals have been fed with organic and inorganic phosphorus compounds and the total intake and output of phosphorus estimated, together with that of nitrogen in most cases. The conclusions which were drawn from the general condition of the animal at the end of the time, and from the effect of the phosphorus compounds upon the nitrogen metabolism, were summarised by F. C. Cook in 1909. They led to the belief that organic phosphorus compounds are directly assimilated and are preferable to inorganic phosphates as the source of phosphorus for animals. Against this belief the recent work of Fingerling [1912; 1, 2] shows that the animal organism can synthesise organic phosphorus compounds from inorganic phosphates. He fed ducks for long periods on food containing only organic phosphorus, or only inorganic phosphorus, as the source of phosphorus. Both sets of ducks laid approximately the same number of eggs and the lecithin and nuclein content of the eggs was about the same in either case. He also analysed the milk of goats which were fed with organic and inorganic phosphorus in the same way as he fed the ducks. In both cases the composition of the milk was the same. Gregersen [1911] has also found that the organic phosphorus compounds can be built up in the animal body from inorganic phosphate, thus confirming the work of Fingerling.

Both organic and inorganic phosphorus compounds can therefore be utilised for the formation of the organic phosphorus compounds in animals; the feeding experiments with organic phosphorus compounds do not show in what form the phosphorus is assimilated, i.e. whether it is absorbed in an organic form or whether it is first hydrolysed and then resynthesised. Phosphorus is excreted in the urine of animals almost entirely as inorganic P_2O_5, whether the intake be organic or inorganic; only a small quantity is excreted in an organic form [Mathison, 1909; Plimmer, Dick and Lieb, 1909; and Gregersen, 1907], and the composition of this organic phosphorus compound is unknown although it is stated to be glycerophosphoric acid. The phosphorus in the faeces is also mainly inorganic P_2O_5. These facts point to the hydrolysis or decomposition of organic phosphorus compounds in the animal organism.

It seemed to me that the only possible means by which the metabolism and assimilation of the natural organic phosphorus compounds could be studied was to ascertain if these compounds were hydrolysed by the enzymes of the digestive tract. The action of the enzymes of the pancreas, intestine and liver upon the various organic phosphorus compounds has therefore been investigated and in addition the action of some plant enzymes has been included so as to obtain a complete and comparative survey of the whole series of organic phosphorus compounds.

I. Glycerophosphoric acid.

The literature on phosphorus metabolism in plants contains no statement as to the hydrolysis or synthesis of glycerophosphoric acid or of lecithin, and until quite recently the knowledge of the metabolism of these substances in animals was equally indefinite. The experiments made by Marfori [1905], Bülow [1894], Mathison [1909], Plimmer, Dick and Lieb [1909] and others had shown that glycerophosphoric acid was completely absorbed when introduced into the body either by the mouth or subcutaneously, and that the phosphorus appeared in the urine as inorganic phosphate and not as glycerophosphoric acid. At the time when these investigations were made[1] no one had tried if the organs of the animal body were able to hydrolyse glycerophosphoric acid and convert it into glycerol and phosphoric acid. It was generally assumed that glycerophosphoric acid was either assimilated as such, or that, since it is an ester, it was hydrolysed like the fats by the lipase in the liver, pancreas and other organs. In 1912 Grosser and Husler [1912] first published experiments which proved that an enzyme (glycerophosphatase) able to hydrolyse glycerophosphoric acid was present in certain animal tissues. They found that the kidney and intestine could completely hydrolyse glycerophosphoric acid, that the lung hydrolysed 62 per cent. and the liver 16 per cent. The spleen, blood and muscle did not hydrolyse glycerophosphoric acid.

The fact that glycerophosphatase is present in the intestine, kidney and lung but not in the pancreas and liver suggests that the hydrolysis is not effected by lipase since the pancreas and liver are the chief animal tissues which contain lipase, whereas the other organs mentioned only contain small amounts of this enzyme.

An enzyme which can hydrolyse glycerophosphoric acid is also present in plants, especially in castor oil seeds. Like the lipase in these seeds it is only

[1] The results of my investigations upon the metabolism of glycerophosphoric acid were communicated to the Biochemical Club at their meeting in July 1911.

liberated from a precursor by the action of dilute acids, but unlike the lipase it is soluble in water [Connstein, Hoyer and Wartenberg, 1902; Armstrong and Ormerod, 1906; Tanaka, 1910]. The action of the two enzymes is thus distinct although it has been impossible to prove this absolutely, as after repeated extraction with water the final extract always contained some glycerophosphatase.

Extracts of yeast and of bran, which contain the enzymes hexosephosphatase [Harden and Young, 1908] and phytase [Suzuki and Takaishi, 1907] respectively, are also able to hydrolyse glycerophosphoric acid.

Experimental.

The general method for determining the action of extracts of various tissues upon glycerophosphoric acid has been to mix a known volume of the extract with either a known volume of a solution of glycerophosphoric acid previously neutralised with soda, or with a solution of sodium glycerophosphate; to withdraw a sample of known volume immediately after mixing and after subsequent intervals of time; and to estimate, after filtering if necessary, the inorganic phosphate in these samples by precipitation with ammonium magnesium citrate in the presence of ammonia, and conversion into magnesium pyrophosphate. By the use of magnesium citrate instead of magnesia mixture the precipitation of organic matter with the ammonium magnesium phosphate is avoided, as was shown by Plimmer and Bayliss in 1906. The total phosphorus content of the mixture was estimated in another portion by Neumann's method as modified by Plimmer and Bayliss [1906]. All results were then calculated for the same volume. In some cases a control experiment consisting of the same volumes of water and of extract was carried out simultaneously. Toluene was added as an antiseptic and the mixtures were kept at 37° C. after the removal of the first sample.

The commercial preparations of glycerophosphoric acid and of sodium glycerophosphate, which are synthetically prepared, were employed. The synthetical compound except for its optical activity is apparently identical with the natural compound obtained from lecithin. Both products seem to be mixtures [Willstätter and Lüdecke, 1904; Power and Tutin, 1905; Tutin and Hann, 1906].

It was originally supposed that the glycerophosphoric acid, since it is an ester, would be hydrolysed by the lipase of the pancreas and other tissues: for this reason the preparations and extracts of tissues used have been purposely made by the usual methods for obtaining an active lipase.

The action of the pancreas was investigated with :

1. The residue from an aqueous extract of trypsin (Fairchild); such residues have been shown by Dietz [1907] to be very strongly lipoclastic in their action. The residue was suspended in a solution of sodium glycerophosphate and the inorganic P_2O_5 was estimated after filtering off and washing the precipitate.

2. A glycerol extract of pig's pancreas prepared by extracting the organ for 24–36 hours with ten times its bulk of a mixture consisting of 90 parts of glycerol and 10 parts of 1 per cent. sodium carbonate, and then straining through muslin. In one experiment this extract was used in aqueous solution, in another in about 50 per cent. glycerol solution. The mixture containing the extract of sodium glycerophosphate was then made just alkaline to phenolphthalein and maintained at 37° C. in the presence of toluene. The samples from the glycerol solution were diluted with water and filtered, and inorganic P_2O_5 was estimated in the filtrate and washings.

3. Fresh pancreatic juice which is known to contain an active lipase. The pancreatic juice in one experiment was kindly collected for me by Prof. Starling, in another experiment by Prof. Bayliss; in the first experiment the juice was activated by enterokinase by adding a small amount of intestinal extract; in the second experiment the juice was not activated.

The action of the liver was investigated with :

1. An extract of dog's liver made by the method of Loevenhart and Peirce [1906] for obtaining an active lipase.

2. An extract of calf's liver prepared according to the method of McCollum and Hart [1908] in their study of phytase in animal tissues. The samples removed at intervals were acidified with a drop of glacial acetic acid, and were boiled and filtered. The coagulum was thoroughly washed and inorganic P_2O_5 was estimated in the filtrate and washings.

The action of the small intestine was investigated by extracts made from the mucous membrane, which was ground up with sand and extracted for 24–48 hours with water containing toluene as antiseptic; and then strained through lint or muslin. In the earlier experiments this extract which was milky in appearance was used directly; the samples, which were removed at intervals, were acidified with acetic acid; the precipitate so formed was filtered off and thoroughly washed and the inorganic P_2O_5 estimated in the clear filtrate and washings. In the later experiments the aqueous extract of the mucous membrane was acidified with acetic acid, and filtered from the precipitate: the clear extract so obtained was found to be active, either when still acid, or after neutralising with sodium carbonate. These clear extracts

were used in all the later experiments with the other organic phosphorus compounds.

The action of the kidney and liver was investigated with extracts made by grinding the organ with sand, and extracting the mass with water, to which toluene was added, for 1–2 days. In the first experiment with the kidney the extract was acidified with acetic acid, the filtrate neutralised with sodium carbonate and used after again filtering. In the other experiments the milky extract was used; each sample was acidified and diluted with the same volumes of acetic acid and water. Inorganic phosphate was estimated in a measured volume of the clear filtrate.

A suspension of shelled castor oil seeds in dilute acetic acid was used for the detection of a glycerophosphatase, samples being removed and the inorganic P_2O_5 estimated in the filtrate and washings. A suspension was also employed when the seed was treated with acid, washed and the extracted residue tested for glycerophosphatase.

Extracts of castor oil seeds were prepared by treating the shelled seeds ground as finely as possible, sometimes after the fat had been previously removed by extraction with ether, with water or with decinormal acetic acid for 1–2 days in the proportion of 5–20 grams of seed and 100–300 c.c. of the acetic acid. A clear filtrate was obtained on filtration. These filtrates were used directly and in some experiments after neutralisation of the acid extract with sodium carbonate.

Preliminary experiments to ascertain if a glycerophosphatase were present in yeast were made with an aqueous extract of pressed yeast prepared in the presence of toluene and by a suspension of zymin; in the latter case the estimations of inorganic P_2O_5 were made in the filtrate and washings from the insoluble matter.

The later and all subsequent experiments with yeast enzymes were made with extracts of the commercial "zymin" of Schroder and Co. of Munich. These extracts were prepared by treating the powder with ten times its weight of water for 24–48 hours in the presence of toluene and then filtering. A pale brown solution resulted.

By simply covering wheat bran with five times its weight of water in the presence of toluene a clear and active extract was always obtained on filtering. The bran extract if kept without contact with air was of a pale brown colour, but when it was exposed to the air and filtered it became rapidly darker in colour.

The following are the analytical data:

Pancreas.

(1) Residue from 1·5 gm. trypsin (Fairchild) suspended in 500 c.c. water containing 5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0121
After 31 days	0·0115
Total	0·1712

(2) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate in water. Mixture made alkaline to phenolphthalein and volume diluted with water to 500 c.c.

	P_2O_5 in gm.
At commencement	0·0128
After 9 days	0·0135
Total	0·1775

(3) 100 c.c. glycerol extract of pig's pancreas + 5 gm. sodium glycerophosphate dissolved in 200 c.c. glycerol + 200 c.c. water and mixture made alkaline to phenolphthalein.

	P_2O_5 in gm.
At commencement	0·0120
After 8 days	0·0126
Total	0·1712

(4) 20 c.c. glycerophosphoric acid diluted to 500 c.c. with water and neutralised with caustic soda + 20 c.c. pancreatic juice of dog + 4–5 drops intestinal juice. Total volume made up to 1000 c.c.

	P_2O_5 in gm.		P_2O_5 in gm.
At commencement	0·0126	After 8 days	0·0759
After 1 day	0·0517	,, 10 ,,	0·0799
,, 2 days	0·0569	,, 13 ,,	0·0824
,, 3 ,,	0·0633	,, 20 ,,	0·0891
,, 5½ ,,	0·0723	,, 36 ,,	0·1110
Total	0·2606		

(5) 2·5 gm. sodium glycerophosphate in water + 50 c.c. pancreatic juice of dog. Total volume made up to 500 c.c.

	P_2O_5 in gm.
At commencement	0·0135
After 14 days	0·0124
Total	0·1661

Liver.

(1) A. 100 c.c. extract of dog's liver by Loevenhart and Peirce's method + 400 c.c. H_2O. B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

	P_2O_5 in gm.	
	A	B
At commencement	0·0006	0·0147
After 1 day ...	0·0013	0·0165
,, 3 days ...	—	0·0157
,, 5 ,, ...	—	0·0122
Total	0·0177	0·1889

(2) A. 125 c.c. extract of calf's liver by McCollum and Hart's method + 125 c.c. water. B. 125 c.c. same extract + 125 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0232	0·0529	0·0297
After 5 days...	0·0339	0·0679	0·0340
Total	0·0659	0·2460	0·1801

Small Intestine.

(1) A. 100 c.c. milky extract of dog's intestine + 400 c.c. water. B. 100 c.c. same extract + 400 c.c. water containing 5 gm. sodium glycerophosphate.

	P_2O_5 in gm.	
A	B	Difference
At commencement:		
0·0042	0·0444	0·0402
After 1 day:		
0·0062	0·1506	0·1444
After 2 days:		
0·0061	0·1539	0·1478
After 6 days:		
0·0061	0·1539	0·1478
Total:		
0·0080	0·1864	0·1784

(2) 80 c.c. clear acid extract of cat's intestine + 5 gm. sodium glycerophosphate in water made up to 500 c.c. with water.

P_2O_5 in gm.	
At commencement	0·0256
After 17 hours ...	0·0313
,, 24 ,, ...	0·0346
,, 5 days ...	0·0680
,, 12 ,, ...	0·0963
Total	0·1458

(3) 80 c.c. clear acid extract of intestines of 2 cats neutralised with soda + 2·5 gm. sodium glycerophosphate in water made up to 250 c.c. with water.

P_2O_5 in gm.	
At commencement	0·0236
After 1 day ...	0·0877
,, 4 days ...	0·1176
,, 9 ,, ...	0·1270
Total	0·1496

Kidney.

(1) A. 100 c.c. clear acid extract of dog's kidneys neutralised with soda + 150 c.c. water. B. 100 c.c. same neutralised extract + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.	
A	B	Difference
At commencement:		
0·0080	0·0090	0·0010
After 3 days:		
0·0075	0·0763	0·0688
After 10 days:		
0·0073	0·0838	0·0765
Total:		
0·0076	0·1661	0·1585

(2) A. 200 c.c. extract of sheep's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
A	B
At commencement:	
0·0256	0·1300
After 1 day:	
0·0321	0·2085
After 4 days:	
0·0332	0·2092
Total:	
0·0406	0·2130

(3) A. 200 c.c. extract of dog's kidneys + 50 c.c. water. B. 200 c.c. same extract + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

P_2O_5 in gm.	
A	B
At commencement:	
0·0071	0·0176
After 1 day:	
0·0084	0·1204
After 5 days:	
0·0092	0·1723
Total:	
0·0241	0·1750

Lung.

A. 200 c.c. extract of dog's lungs + 50 c.c. water. B. 200 c.c. extract of dog's lungs + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement	0·0069	0·0148	0·0079
After 1 day ...	0·0110	0·0344	0·0234
,, 2 days ...	0·0128	0·0472	0·0344
,, 4 ,, ...	0·0161	0·0730	0·0569
Total	0·0241	0·2282	0·2041

Castor oil seeds.

(*a*) 5 gm. ground seeds suspended in 250 c.c. $\frac{N}{10}$ acetic acid containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0089
After 1 day ...	0·1199
,, 2 days ...	0·1395
,, 3 ,, ...	0·1538
,, 8 ,, ...	0·1852
Total	0·2308

(*b*) 60 c.c. aqueous extract of ground seeds + 200 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0121
After 12 days ...	0·0105
Total	——

(*c*) 130 c.c. $\frac{N}{10}$ acetic acid extract + 120 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0217
After 1 day ...	0·0888
,, 2 days ...	0·1027
,, 3 ,, ...	0·1109
,, 6 ,, ...	0·1204
Total	0·2143

(*d*) 100 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0356
After 1 day ...	0·0920
,, 2 days ...	0·1008
,, 7 ,, ...	0·1179
,, 12 ,, ...	0 1263
Total	0·2003

50 c.c. extract contained—

At commencement	0·0477 gm.	inorg. P_2O_5
After 12 days ...	0·0504 ,,	,,
Total P_2O_5 ...	0·0520 gm.	

Increases are thus due entirely to hydrolysis of the glycerophosphate.

(*e*) 200 c.c. $\frac{N}{10}$ acetic acid extract of ground seeds freed from fat by extraction with ether and kept for several months so as to convert all organic phosphorus into inorganic phosphate + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0410
After 1 day ...	0·0579
,, 2 days ...	0·0711
,, 3 ,, ...	0·0806
,, 6 ,, ...	0·1030
Total	0·2003

(*f*) Residue of seeds in expt. (*c*) thoroughly washed by decantation and filtration suspended in 250 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0165
After 3 days ...	0·0651
,, 10 ,, ...	0·0889
,, 20 ,, ...	0·1011
Total	0·1826

(*g*) Residue of seeds in expt. (*e*) after thorough washing was suspended in 250 c.c. of 1 per cent. solution of sodium glycerophosphate in water.

	P_2O_5 in gm.
At commencement	0·0005
After 2 days ...	0·0259
,, 4 ,, ...	0·0338
,, 7 ,, ...	0·0522
Total	0·1500

Yeast.

(*a*) 80 c.c. extract of yeast + 170 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·0396
After 1 day ...	0·0776
„ 3 days ...	0·0869
„ 7 „ ...	0·0952
Total	0·2244

50 c.c. yeast extract contained—
0·0804 gm. inorg. P_2O_5
0·0930 gm. total P_2O_5

Increases are therefore due to hydrolysis of glycerophosphate.

(*b*) A. 10 gm. zymin suspended in 250 c.c. water. B. 10 gm. zymin suspended in 250 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0215	0·0847	0·0632
After 1 day ...	0·0527	0·1323	0·0796
„ 2 days	0·0582	0·1424	0·0842
„ 6 „	0·0632	0·1539	0·0907
Total	0·0850	0·2637	0·1787

(*c*) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement	0·0304	0·0774	0·0470
After 1 day ...	0·0341	0·0907	0·0566
„ 8 days ...	0·0379	0·1068	0·0689
Total	0·0621	0·2409	0·1788

Bran.

(1) 200 c.c. extract of bran + 50 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.
At commencement	0·1430
After 1 day ...	0·1696
„ 2 days ...	0·1839
„ 4 „ ...	0·1877
Total	0·2904

(2) A. 125 c.c. extract of bran + 125 c.c. water. B. 125 c.c. extract of bran + 125 c.c. water containing 2·5 gm. sodium glycerophosphate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0374	0·0389	0·0015
After 2 days	0·0389	0·0731	0·0342
„ 4 „	0·0389	0·0852	0·0463
„ 9 „	0·0393	0·1014	0·0621
Total	0·0406	0·1965	0·1559

The gradual increase in the amount of inorganic P_2O_5, which occurred when the extracts of intestinal mucosa, kidney, castor oil seeds, yeast and bran were tested, is the best evidence that the hydrolysis of glycerophosphoric acid has been effected by an enzyme present in the tissues. The enzyme is not present in the pancreas and liver of animals. Though an increase in the amount of inorganic phosphate was found in the experiment with pancreatic juice activated by 4–5 drops of intestinal juice, the increase takes place very slowly; one-fifth is hydrolysed in 2 to 3 days, and after 36 days the hydrolysis is less than one-half of the total P_2O_5 in the solution. An explanation of

this increase is given by the experiments with intestinal extract, which contains the active enzyme; a small amount of the enzyme had therefore been added when activating the pancreatic juice. The absence of the enzyme from the extracts of pancreas and liver, which were prepared specially for lipase, and the presence of the enzyme in the extracts of the intestine and kidney, tissues not usually regarded as good sources of lipase, point to this enzyme being distinct from lipase. Proof of the difference of the enzymes is given by the experiment with castor oil seeds; lipase is insoluble in water but an aqueous extract contains glycerophosphatase. Its presence in bran, in which lipase has not yet been shown, is further proof that the two enzymes are not identical. A comparison of the actual amounts of the enzymes present in the several tissues is not possible, but it may be noticed that the amount of glycerophosphatase in the lung is very much less than in the intestine and kidney. The amount in yeast is also small and the amount contained in bran is smaller still.

II. Phytic acid.

Phytin, the calcium magnesium salt of inositolphosphoric acid, or phytic acid, was first isolated by Posternak in 1903 from various seeds in which it is present as a reserve material containing phosphorus in organic combination. It also occurs in the bran of cereals [Hart and Andrews, 1903], that of rice being particularly rich in it [Suzuki and Yoshimura, 1907]. Phytin is thus the most important phosphorus-containing foodstuff for herbivora, and it has been administered to man to supply organically combined phosphorus. On this account more work has been done upon its metabolism than upon that of any of the other organic phosphorus compounds.

It has been shown by Scofone [1905], Giascosa [1905], Mendel and Underhill [1906], and also Horner [1907] that phytin, when given to animals by the mouth, is absorbed and that the phosphorus is excreted as inorganic phosphorus in the urine; but the absorption is never complete.

Suzuki and Takaishi [1907] have found that bran contains an enzyme, phytase, which completely hydrolyses phytin into inositol and phosphoric acid; Jordan, Hart and Patten [1906] have shown that the enzymes, pepsin and trypsin, of the animal body have no action on phytin, but according to McCollum and Hart [1908], the liver and also the blood contain a phytin-splitting enzyme.

The hydrolysis of glycerophosphoric acid by the enzymes of the intestinal mucosa suggested that phytic acid might also be split up by this tissue. Experiment, however, showed the contrary. Neither the mucous membrane

of the intestine of the carnivora, dog and cat, nor that of the herbivora, sheep, ox and rabbit, even when the latter animal was fed for some weeks on bran, contained an enzyme which was able to hydrolyse phytic acid. Phytic acid thus behaved very differently from glycerophosphoric acid. The difference in the behaviour of the two compounds does not extend to the enzymes of the liver. The hydrolysis of phytic acid by the liver observed by McCollum and Hart was very small and it has been impossible to regard this as a hydrolysis when compared with other results. Phytic acid is thus not hydrolysed by the enzymes of the digestive tract of animals.

The enzymes in an extract of zymin have no action upon phytin, but it is slowly hydrolysed by an acid extract of castor oil seeds. The only enzyme which readily hydrolyses phytin is contained in an extract of bran.

Experimental.

Commercial phytin was employed as the source of phytic acid in these experiments. This substance except for a small residue is easily soluble in water, to which it gives a distinctly acid reaction to litmus, and from which it is precipitated on rendering the solution alkaline with ammonia. It contains a small quantity of inorganic phosphate. The calcium was precipitated from the aqueous solution of phytin by adding the calculated quantity of oxalic acid, or of sodium or potassium oxalate. The solution of the free acid, or of the sodium or potassium salt, obtained on filtering was used as substrate in the experiments.

Extracts of the various tissues were prepared as described under glycerophosphoric acid; the mixture of enzyme solution and phytate was kept at 37° C. in the presence of toluene. Samples were removed at intervals and the inorganic P_2O_5 estimated.

In the first experiments the estimation of inorganic phosphate was carried out by precipitation with ammonium magnesium citrate in the presence of ammonia, but it was soon observed that the inorganic phosphate in the presence of phytic acid was not immediately precipitated but only came down slowly after standing for some time, and that the filtrate frequently gave a further precipitate on standing. The precipitate did not have the usual appearance of ammonium magnesium phosphate and it adhered very tenaciously to the sides of the vessel in which the precipitation took place. On converting these precipitates by heating into the pyrophosphate the weights obtained were also very variable. An examination into the cause of these difficulties revealed the fact that phytic acid inhibits the precipitation

of inorganic phosphate as ammonium magnesium phosphate and that under certain conditions phytic acid also separates as magnesium phytate from ammoniacal solution.

Hart and Andrews [1903], in their study of the relative amounts of inorganic and organic phosphate in foodstuffs, precipitated the inorganic phosphate as ammonium phosphomolybdate at 65° C. in the presence of dilute nitric acid. This method was therefore used but the precipitation was carried out at room temperature since it was thought that the phytic acid might be hydrolysed by the nitric acid at 65° C. A slight hydrolysis does occur at this temperature (see the following paper), but it is insufficient to vitiate the results of Hart and Andrews, or those of McCollum and Hart, who employed this method. The precipitation of the ammonium phosphomolybdate at the ordinary room temperature takes place slowly, but is generally complete in 1–2 days. The completion of the precipitation is shown by the colour of the solution; it is yellow when the reagents are added and becomes quite colourless when the deposition of the yellow precipitate is finished. In the presence of organic matter the solution may remain yellow, which is due to the action of nitric acid on protein in the enzyme extracts. At the same time protein matter is precipitated so that the estimation cannot be carried out by titration as in Neumann's method. When the precipitation was complete the precipitate was filtered off and washed with water and then dissolved in dilute caustic soda. Inorganic phosphate was then precipitated by ammonium magnesium citrate. The details of these experiments will be described in a later paper dealing with the analysis of phytin. Total P_2O_5 was estimated by Neumann's method.

Pancreas.

130 c.c. pancreatic juice + 130 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0025
After 14 days ...	0·0055
Total	0·0850

Liver.

A. 125 c.c. extract of dog's liver + 125 c.c. H_2O. B. 125 c.c. extract of dog's liver + 125 c.c. sodium phytate solution.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0348	0·0408	0·0060
After 1 day ...	0·0351	0·0454	0·0103
„ 5 days...	0·0379	0·0486	0·0107
„ 12 „ ...	0·0376	0·0516	0·0140
Total	0·0469	0·1407	0·0938

Intestine.

(1) 50 c.c. extract of cat's intestine (which hydrolysed trypsin digest of caseinogen) + 200 c.c. phytin solution.

	P_2O_5 in gm.
At commencement	0·0126
After 1 day ...	0·0145
,, 2 days ...	0·0158
,, 4 ,, ...	0·0180
Total	0·1154

(2) 100 c.c. extract of dog's intestine + 150 c.c. phytin solution.

	P_2O_5 in gm.
At commencement	0·0190
After 5 days ...	0·0179
Total	0·1116

(3) 150 c.c. extract of dog's intestine + 100 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0256
After 1 day ...	0·0264
,, 2 days ...	0·0283
,, 9 ,, ...	0·0306
Total	0·1154

(4) 200 c.c. extract of ox intestine + 100 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0198
After 1 day ...	0·0219
,, 3 days ...	0·0251
,, 5 ,, ...	0·0264
,, 8 ,, ...	0·0277
Total	0·0964

(5) 150 c.c. extract of sheep's intestine + 120 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0137
After 6 days ...	0·0139
Total	0·0913

(6) 100 c.c. extract of rabbit's intestine + 150 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0085
After 6 days ...	0·0106
Total	0·0964

(7) 150 c.c. extract of intestine of rabbit fed for 3 weeks on bran + 100 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0188
After 2 days ...	0·0267
,, 5 ,, ...	0·0299
,, 8 ,, ...	0·0301
Total	0·1065

Castor oil seeds.

(*a*) 150 c.c. acetic acid extract + 100 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0134
After 1 day ...	0·0190
,, 2 days ...	0·0230
,, 4 ,, ...	0·0291
Total	0·1103

(*b*) 100 c.c. acetic acid extract + 150 c.c. sodium phytate solution.

	P_2O_5 in gm.
At commencement	0·0188
After 1 day ...	0·0279
,, 4 days ...	0·0458
,, 8 ,, ...	0·0554
,, 12 ,, ...	0·0633
Total	0·1458

Yeast.

(1) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0291	0·0362	0·0071
After 7 days...	0·0362	0·0412	0·0050
Total	0·0583	0·1509	0·0926

(2) A. 100 c.c. extract of zymin + 150 c.c. water. B. 100 c.c. extract of zymin + 150 c.c. sodium phytate solution.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0330	0·0373	0·0043
After 9 days...	0·0359	0·0411	0·0052
Total	0·0579	0·1420	0·0841

Bran.

A. 125 c.c. extract + 125 c.c. water. B. 125 c.c. extract + 125 c.c. sodium phytate solution.

	P_2O_5 in gm.		
	A	B	Difference
At commencement	0·0513	0·0566	0·0053
After 1 day ...	0·0531	0·1087	0·0556
„ 4 days ...	0·0559	0·1354	0·0795
„ 10 „ ...	0·0559	0·1354	0·0795
Total	0·0609	0·1510	0·0901

These figures show such a marked difference in the extent of hydrolysis by the extract of bran and by the other enzymes that it can scarcely be concluded that phytase exists in any of the extracts except in that of castor oil seeds; the hydrolysis in this case is very slow; only a very slight increase in the amount of inorganic phosphate is noticeable with the intestinal and liver extracts, and none with pancreatic juice and extract of zymin.

III. Hexosephosphoric acid.

Harden and Young [1908] have found that hexosephosphoric acid is formed during the fermentation of glucose and fructose by yeast or by zymase, and that it is subsequently hydrolysed. They attribute the hydrolysis to a special enzyme, hexosephosphatase. Harding [1912] has recently investigated the action of enzymes upon hexosephosphate and has observed that an enzyme present in castor oil seeds hydrolyses it, and that this enzyme is not present in ox pancreas. These data are so closely parallel to those obtained with glycerophosphoric acid that it was very important to test the action of the intestine and of bran upon this compound. Extracts of both these tissues were found to contain hexosephosphatase. The parallelism between the action of glycerophosphatase and hexosephosphatase is thus extremely close, and it would seem that the same phosphatase hydrolyses both these compounds.

Experimental.

The hexosephosphoric acid employed in these two experiments was most kindly supplied to me by Dr Young. The solution of hexosephosphoric acid was neutralised and mixed with a neutral extract of intestinal mucous membrane and extract of bran, both of which were prepared by the methods described under glycerophosphoric acid. Toluene was added and the mixture kept at 37° C. The inorganic P_2O_5 was estimated in samples by precipitation

with ammonium magnesium citrate and converted into magnesium pyrophosphate. Total P_2O_5 in the solution was estimated by Neumann's method with another sample.

(*a*) *Intestinal Extract.*

100 c.c. extract + 160 c.c. hexosephosphate solution.

	P_2O_5 in gm.
At commencement	0·0435
After 2 days	0·0815
„ 4 „	0·0819
Total	0·0860

P_2O_5 in intestinal extract added per 50 c.c. sample = 0·0146 gm.

(*b*) *Bran Extract.*

A. 100 c.c. bran extract + 150 c.c. water. B. 100 c.c. hexosephosphate solution + 150 c.c. water. C. 100 c.c. bran extract + 100 c.c. hexosephosphate solution + 50 c.c. water.

	A Bran extract alone	B Hexosephosphate alone	C Hexosephosphate + bran extract	Difference between C and A + B
At commencement	0·0225	0·0092	0·0352	0·0035
After 1 day ...	0·0236	0·0093	0·1006	0·0677
„ 2 days ...	0·0240	0·0110	0·1115	0·0765
„ 4 „ ...	0·0244	0·0121	0·1188	0·0823
Total	0·0279	0·1801	0·2054	—

Hydrolysis has occurred with both intestinal extract and bran extract; it is most marked in the case of intestinal extract where it was complete in two days. Bran extract caused a marked hydrolysis in one day but it was not complete in four days, in which time scarcely half of the compound was hydrolysed.

IV. Ethyl phosphoric acid and diethyl phosphoric acid.

The action of enzymes upon the simple esters of phosphoric acid has never been investigated. Ethyl phosphoric acid and diethyl phosphoric acid were therefore tested against those extracts of tissues which had been found to hydrolyse glycerophosphoric acid, phytic acid and hexosephosphoric acid. The parallelism in the action of the enzyme upon glycerophosphoric and hexosephosphoric acid occurred again in their action upon ethyl phosphoric acid; diethyl phosphoric acid was not hydrolysed by intestinal extract nor by extract of castor oil seeds; unfortunately the quantity of diethyl phosphoric acid available was not sufficient to test against the other extracts.

The material used in these investigations was prepared either by the

action of phosphoric pentoxide or of phosphorus oxychloride upon absolute alcohol. The product of the reaction in each case was neutralised with excess of baryta; barium phosphate was filtered off and the clear filtrate precipitated by an equal volume of alcohol; barium ethyl phosphate was precipitated in crystalline form and recrystallised from water and alcohol. This was converted into the sodium salt by treatment with the calculated quantity of sodium sulphate and the solution so obtained was used in the experiments with the enzymes. A small quantity of barium diethyl phosphate was contained in the alcoholic filtrate obtained in the preparation by means of phosphorus pentoxide; it crystallised out on concentration of the alcoholic solution and was purified by recrystallisation from dilute alcohol. The solution of the sodium salt prepared by treatment of the barium salt with sodium sulphate was employed for the investigation of the action of the enzymes.

Experimental.

The enzyme solutions were prepared in the same way as described under glycerophosphoric acid. Inorganic phosphate was estimated by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. The experiments were the following:—

A. *Sodium Ethyl Phosphate.*

Pancreas.

0·5 gm. trypsin (Fairchild) was dissolved in 110 c.c. deci-normal sodium carbonate solution and filtered. 100 c.c. extract + 200 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0·0052
After 11 days ...	0·0046
Total	0·0659

Intestine.

(1) 125 c.c. neutral intestinal extract + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0·0168
After 1 day ...	0·0492
,, 2 days ...	0·0572
,, 4 ,, ...	0·0628
Total	0·0654

(2) 110 c.c. neutral intestinal extract + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0·0121
After 1 day ...	0·0457
,, 2 days ...	0·0560
,, 4 ,, ...	0·0626
Total	0·0672

Castor oil seeds.

(1) 125 c.c. acetic acid extract of seeds + 130 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0·0070
After 1 day ...	0·0235
,, 2 days ...	0·0307
,, 4 ,, ...	0·0383
Total	0·0697

(2) 110 c.c. neutralised extract of castor oil seeds + 150 c.c. sodium ethyl phosphate solution.

P_2O_5 in gm.	
At commencement	0·0075
After 1 day ...	0·0135
,, 4 days ...	0·0177
Total	0·0735

Yeast.

60 c.c. extract of zymin + 200 c.c. sodium ethyl phosphate solution.

	P_2O_5 in gm.
At commencement	0·0111
After 1 day ...	0·0283
„ 5 days ...	0·0398
„ 12 „ ...	0·0491
Total	0·1052

Bran.

60 c.c. extract of bran + 200 c.c. sodium ethyl phosphate solution.

	P_2O_5 in gm.
At commencement	0·0148
After 1 day ...	0·0190
„ 2 days ...	0·0216
„ 5 „ ...	0·0259
„ 12 „ ...	0·0319
Total	0·0938

B. *Sodium Diethyl Phosphate.*

Intestine.

150 c.c. acid extract of intestinal mucosa + 100 c.c. sodium diethyl phosphate solution: after 3 days solution neutralised with a few drops of sodium carbonate solution.

	P_2O_5 in gm.
At commencement	0·0250
After 13 days ...	0·0250
Total	0·0888

Castor oil seeds.

150 c.c. neutralised acid extract + 100 c.c. sodium diethyl phosphate solution: after 4 days acidified with 2 drops of glacial acetic acid.

	P_2O_5 in gm.
At commencement	0·0138
After 11 days ...	0·0156
Total	0·0875

Sodium ethyl phosphate is hydrolysed most readily by the extract of the intestinal mucosa and it is hydrolysed by an acid extract of castor oil seeds; the hydrolysis also takes place where the extract is neutralised.

Extracts of yeast and of bran also contain an enzyme which can hydrolyse it; in the case of bran the hydrolysis is very slow.

Sodium diethyl phosphate was not hydrolysed by either intestinal extract or extract of castor oil seeds.

V. Hydroxymethyl-phosphinic acid ($OH \cdot CH_2 \cdot PO_3 \cdot H$).

The previous results of the action of enzymes upon compounds which are esters of phosphoric acid led to an investigation of their action upon hydroxymethyl-phosphinic acid, a compound in which the phosphoric acid is linked to carbon instead of through oxygen as in the other compounds.

This compound was prepared by Mr H. J. Page [1912] in this laboratory and a quantity was kindly placed at my disposal for this purpose. No natural organic phosphorus compounds of this constitution are known; these experiments were consequently of further interest, since if hydroxymethyl-phosphinic acid were hydrolysed by enzymes, natural organic compounds of this constitution might be expected to occur in nature.

Experimental.

The solution of the free acid was exactly neutralised and mixed with the enzyme solution which was prepared as previously described; the analysis was carried out in the same way as with glycerophosphoric acid.

The results were:

(*a*) *Action of Trypsin.*

105 c.c. $\frac{N}{10}$ sodium carbonate extract of 0·5 gm. trypsin (Fairchild) + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.
At commencement	0·0046
After 7 days ...	0·0055
Total	0·1344

(*b*) *Action of Extract of Intestinal Mucosa.*

100 c.c. neutralised extract of dog's intestinal mucosa + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.
At commencement	0·0134
After 4 days ...	0·0144
Total	0·2422

(*c*) *Action of Extract of Castor oil seeds.*

100 c.c. acetic acid extract of castor oil seeds + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.
At commencement	0·0009
After 4 days ...	0·0019
Total	0·2840

(*d*) *Action of Extract of Bran.*

100 c.c. extract of bran + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.
At commencement	0·0508
After 4 days ...	0·0526
Total	0·2244

(*e*) *Action of Extract of Zymin.*

(1) 100 c.c. extract of zymin + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.
At commencement	0·0393
After 1 day ...	0·0487
,, 2 days ...	0·0509
,, 4 ,, ...	0·0528
,, 7 ,, ...	0·0549
Total	0·2333

(2) A. 100 c.c. extract of zymin + 150 c.c. H_2O as control. B. 100 c.c. extract of zymin + 150 c.c. sodium hydroxymethyl-phosphinate solution.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0216	0·0227	0·0011
After 2 days...	0·0296	0·0362	0·0066
,, 5 ,, ...	0·0310	0·0393	0·0083
,, 8 ,, ...	0·0329	0·0424	0·0095
Total	0·0583	0·1977	0·1394

As was expected this compound was not hydrolysed by any of the extracts which contain glycerophosphatase even if we include the action of the extract of zymin; in this case an increase of inorganic P_2O_5 was observed at the commencement but the increase was very slight later on; it is probably due to a more rapid separation of P_2O_5 in the enzyme solution arising from a change in reaction when the mixture was made.

VI. Nucleic acid.

The action of the enzymes of animal organs upon various nucleic acids has been studied by many investigators, but their attention has been confined almost entirely to the separation of the purine bases from the complex molecule, and little or no attention has been given to the separation of the phosphoric acid. Milroy [1896] found that 10–16 per cent. of the total phosphorus in a peptic digest of nuclein was inorganic phosphoric acid, and that in a trypsin digest the amount of inorganic phosphoric acid varied from 6–47 per cent. of the total phosphorus content. No estimation of the amount of the phosphoric acid present in the enzyme solution was made, so that a portion or the whole of the inorganic phosphate might have been so introduced into his solutions. Levene and Medigreceanu [1911] in some of their recent papers state that the enzymes of the intestinal mucosa hydrolyse nucleic acid with the liberation of phosphoric acid; no estimation of the amount of the inorganic phosphate was made. The formation of phosphoric acid during the autolysis of yeast, first observed by Béchamps in 1865, was attributed to the hydrolysis of nucleic acid; hexosephosphate and hexosephosphatase were unknown at that time and it is most probable that the phosphoric acid observed by Béchamps originated from this compound rather than from nucleic acid.

There is thus no satisfactory proof that inorganic phosphate is liberated by enzymes from nucleic acid though it is very probable. The following experiments were therefore carried out to test these earlier observations and to compare the hydrolysis of nucleic acid by the same enzymes which have been found to liberate phosphoric acid from the other organic phosphorus compounds.

It has not been possible to test all the various nucleic acids but the results show that an extract of intestinal mucosa slowly separates inorganic phosphoric acid from thymus nucleic acid and from wheat nuclein, but more rapidly and completely from the organic phosphorus compound (inosinic acid) in meat extract; trypsin does not hydrolyse thymus nucleic acid, nor the compound in meat extract. An extract of zymin showed no appreciable separation of phosphoric acid from the thymus nucleic acid, whereas an extract of bran hydrolysed it.

Experimental.

The thymus nucleic acid used in these experiments was prepared for me by Miss R. F. Skelton by Kossel's method [1894]. The preparation was almost pure white in colour and contained 8 per cent. of phosphorus and

17 per cent. of nitrogen. It dissolved readily in decinormal sodium carbonate giving a solution which was faintly brown in colour.

The meat extract was a solution of lemco, and the wheat nuclein was also kindly prepared for me by Miss Skelton from wheat germ by digestion with pepsin hydrochloric acid. The insoluble residue of nuclein was dissolved in dilute sodium carbonate, separated from starch by centrifugalisation and then reprecipitated by acid. After washing with water it was dissolved in decinormal sodium carbonate solution and the brown solution thus obtained was employed.

The enzyme extracts were prepared in the same way as those used in investigating the hydrolysis of glycerophosphoric acid; the extract was mixed with the solution of substrate and kept in the presence of toluene at 37° C. Samples were removed and inorganic P_2O_5 estimated by precipitation with ammonium magnesium citrate. Total P_2O_5 was estimated in another sample by Neumann's method.

(*a*) *Action of Extract of Pancreas.*

(1) 2 gm. trypsin (Fairchild) were dissolved in 260 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. A. 125 c.c. extract + 125 c.c. water. B. 125 c.c. extract + 125 c.c. solution containing 2·3 gm. thymus nucleic acid dissolved in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.

	A	B
At commencement	0·0071	0·0070
After 1 day ...	0·0074	0·0087
,, 11 days ...	0·0074	0·0089
Total	0·0139	0·0951

(2) 0·5 gm. trypsin (Fairchild) dissolved in 110 c.c. $\frac{N}{10}$ sodium carbonate solution and filtered. 100 c.c. extract + 150 c.c. of a solution containing 10 gm. of lemco.

P_2O_5 in gm.

At commencement	0·1189
After 7 days ...	0·1194
Total	0·1331

(*b*) *Action of Extract of Intestine.*

(1) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 150 c.c. solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

P_2O_5 in gm.

At commencement	0·0152*
After 1 day ...	0·0254
,, 2 days ...	0·0277
,, 4 ,, ...	0·0310
,, 7 ,, ...	0·0327
Total	0·0938

* 0·0117 gm. P_2O_5 was contained in the intestinal extract for each sample.

(2) 100 c.c. neutralised extract of intestinal mucous membrane of dog + 200 c.c. solution of wheat nuclein in sodium carbonate neutralised with acetic acid.

P_2O_5 in gm.

At commencement	0·0147
After 1 day ...	0·0211
,, 2 days ...	0·0200
,, 4 ,, ...	0·0228
Total	0·0596

(3) 60 c.c. neutralised extract of intestinal mucous membrane of a dog + 11 gm. lemco dissolved in 200 c.c. water.

	P_2O_5 in gm.
At commencement	0·1254
After 1 day ...	0·1458
,, 4 days ...	0·1470
Total	0·1521

(4) 100 c.c. neutralised extract of intestinal mucous membrane of cat + 10 gm. lemco dissolved in 160 c.c. water.

	P_2O_5 in gm.
At commencement	0·1409
After 1 day ...	0·1668
,, 5 days ...	0·1712
Total	0·1839

(c) *Action of Extract of Zymin.*

A. 100 c.c. extract of zymin + 150 c.c. H_2O.
B. 100 c.c. extract of zymin + 150 c.c. of a solution containing 2 gm. thymus nucleic acid in $\frac{N}{10}$ sodium carbonate.

	P_2O_5 in gm.		
	A	B	Difference
At commencement ...	0·0216	0·0235	0·0019
After 2 days...	0·0296	0·0322	0·0026
,, 5 ,, ...	0·0310	0·0353	0·0043
,, 8 ,, ...	0·0329	0·0380	0·0051
Total	0·0583	0·1281	0·0698

(d) *Action of Extract of Bran.*

100 c.c. extract of bran + 150 c.c. solution containing 2 gm. thymus nucleic acid in decinormal sodium carbonate.

	P_2O_5 in gm.
At commencement	0·0297*
After 1 day ...	0·0432
,, 2 days ...	0·0498
,, 5 ,, ...	0·0625
Total	0·1091

* 0·0279 gm. was contained in the bran extract for each sample.

Trypsin has no action upon nucleic acid; the action of intestinal extract upon thymus nucleic acid is very slight and is still less upon wheat nuclein; it readily hydrolyses the organic phosphorus compound contained in lemco. Extract of zymin has also a very slight hydrolysing action, but extract of bran is more active. No marked hydrolysis of nucleic acid is noticeable by any of the extracts.

VII. Phosphoprotein.

The separation of the phosphorus of caseinogen by the action of enzymes and by alkali was investigated in 1906 by Plimmer and Bayliss [1906], who confirmed the earlier work of Biffi with regard to the action of trypsin. Pepsin slowly converted the phosphorus of caseinogen into a soluble form, but without the formation of phosphoric acid. Trypsin separated 35 per cent. of the phosphorus as phosphoric acid, the remaining 65 per cent. being present as a soluble organic compound. Papain also converted the phosphorus of caseinogen into a soluble form, but no investigation was made as to whether phosphoric acid was produced.

Complete separation of the phosphorus from caseinogen was thus not effected by trypsin. It seemed however very probable that the enzyme of

the intestinal mucosa, most probably erepsin, which according to Cohnheim hydrolyses caseinogen, would complete the separation as phosphoric acid. This was found to be the case. Papain also separates phosphoric acid from caseinogen in both acid and alkaline media but the separation is very slow. The vegetable proteoclastic enzyme "digestin" prepared by Yenjo in Japan from an *Aspergillus* behaves like papain. Neither an extract of zymin nor an extract of bran separates phosphoric acid from the organic phosphorus compound remaining after the action of trypsin upon caseinogen. It might have been expected that the extract of zymin would hydrolyse this compound and its inactivity is probably accounted for by its method of preparation with acetone, which coagulates the protein to which the proteoclastic enzyme is attached, so that the latter is not extracted by water.

Experimental.

A trypsin digest of caseinogen was prepared by digesting 200 gm. pure caseinogen dissolved in 2000 c.c. water containing 10 c.c. ammonia of sp. gr. 0·880 with 3 gm. trypsin (Fairchild) for two months at 37° in the presence of 20 c.c. of toluene. The solution was no longer alkaline to phenolphthalein after one month and ammonia was added till the solution became just red to this indicator. At the end of this time the tyrosine which had separated was filtered off. The clear brown solution contained 0·0456 gm. total P_2O_5 and 0·0209 gm. inorganic P_2O_5 per 50 c.c. 56 per cent. of the total P_2O_5 was thus present as organic P_2O_5. A portion of this solution which was kept for another four months was found to contain 0·0418 gm. total P_2O_5 and 0·0228 gm. inorganic P_2O_5 per 50 c.c. A year later the amounts were 0·0430 gm. total P_2O_5 and 0·0242 gm. inorganic P_2O_5 i.e. the solution contained 45 per cent. organic phosphorus.

Hydrolysis thus proceeds but even after 18 months it is not complete.

The action of the extracts of the intestinal mucosa, bran and zymin was investigated in the same way as described under glycerophosphoric acid. The action of papain and digestin was investigated with caseinogen which was dissolved in dilute caustic soda solution (20 gm. caseinogen + 40 c.c. semi-normal NaOH + 500 c.c. water): one half of this mixture was used for the experiment in alkaline solution and 200 c.c. of the remainder were acidified with 25 c.c. of semi-normal sulphuric acid and employed for the experiment in acid solution. Samples were removed at intervals and filtered for the estimation of the inorganic P_2O_5 and total P_2O_5. The action of papain was also tested upon the above trypsin digest of caseinogen.

The experiments were:

Action of Intestinal Extract.

(*a*) 125 c.c. extract of cat's intestine + 125 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0·0223
After 3 days ...	0·0500
„ 5 „ ...	0·0492
Total	0·0579

(*b*) 50 c.c. acid extract of cat's intestine + 200 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0·0224
After 18 hours ...	0·0302
„ 24 „ ...	0·0313
„ 42 „ ...	0·0325
„ 4 days ...	0·0338
Total	0·0406

(*c*) 50 c.c. acid extract of dog's intestine + 200 c.c. trypsin digest of caseinogen.

P_2O_5 in gm.	
At commencement	0·0244
After 17 hours ...	0·0329
„ 65 „ ...	0·0357
„ 6 days ...	0·0367
Total	0·0431

Action of Papain.

(*a*) In acid solution on caseinogen.

P_2O_5 in gm.	
After 38 days	0·0037
„ 64 „	0·0052
Total	0·0203

(*b*) In alkaline solution on caseinogen.

P_2O_5 in gm.	
After 38 days	0·0075
„ 64 „	0·0092
Total	0·0349

(*c*) On trypsin digest. 200 c.c. trypsin digest + 55 c.c. extract of papain.

P_2O_5 in gm.	
At commencement	0·0166
After 1 day ...	0·0168
„ 2 days ...	0·0166
„ 3 „ ...	0·0168
Total	0·0368

Action of Digestin (Yenjo).

(*a*) In acid solution on caseinogen.

P_2O_5 in gm.	
After 4 months	0·0124
Total	0·0203

(*b*) In alkaline solution.

P_2O_5 in gm.	
After 4 months	0·0236
Total	0·0330

Action of Extract of Zymin.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P_2O_5 in gm.	A	B	Difference
At commencement ...	0·0286	0·0085	0·0201
After 2 days...	0·0316	0·0131	0·0185
„ 4 „ ...	0·0282	0·0139	0·0143
Total	0·0634	0·0279	0·0355

Action of Extract of Bran.

A. 200 c.c. trypsin digest + 50 c.c. extract.
B. 200 c.c. water + 50 c.c. extract.

P_2O_5 in gm.	A	B	Difference
At commencement ...	0·0338	0·0142	0·0196
After 2 days...	0·0315	0·0148	0·0167
„ 4 „ ...	0·0327	0·0147	0·0180
Total	0·0494	0·0190	0·0304

In comparison with the other extracts that of the intestinal mucosa is the most active in separating phosphoric acid from caseinogen. The hydrolysis is almost complete within 24 hours; in all these experiments a small quantity remained in an organic form. This is most probably due to impurity and corresponds to the small quantity of organic phosphorus which always remains

when phosphoprotein is decomposed by alkali, as was found by Plimmer and Scott [1908].

Papain in alkaline solution separated one quarter of the total P_2O_5 in 35 days and this amount was slightly increased in 64 days; in acid solution in the same time one fifth was separated as inorganic phosphate, which amount was increased to one quarter in 64 days; further separation does not seem to occur as papain does not hydrolyse the organic P_2O_5 present in the trypsin digest of caseinogen. Digestin also never separated the whole of the phosphorus of caseinogen. Neither extract of bran nor extract of zymin separated phosphoric acid from the organic phosphorus in a trypsin digest.

Summary and Conclusions.

The action of enzymes upon the organic phosphorus compounds is best summarised by a tabulation of the results:

	Pancreas	Liver	Intestine	Castor oil seeds	Yeast (Zymin)	Bran
Glycerophosphoric acid ...	0	0	+	+	+	+
Hexosephosphoric ,, ...	0	...	+	+	+	+
Ethyl phosphoric ,, ...	0	...	+	+	+	+
Diethyl phosphoric ,, ...	...	...	0	0	...	...
Phytic acid ...	0	0	0	+	0	+
Nucleic acid (Thymus) ...	0	...	+	...	+?	+
,, ,, (Wheat) ...	...	...	+?	...	...	...
,, ,, (Meat) ...	0	...	+	...	...	...
Hydroxymethylphosphinic acid	0	...	0	0	+?	0
Phosphoprotein	$\frac{2}{3}$	...	+	...	0	0

The most active tissue in the hydrolysis of the organic phosphorus compounds is the intestinal mucosa; all the organic phosphoric compounds except phytic acid were hydrolysed by it. Diethyl phosphoric acid and hydroxymethyl-phosphinic acid must be excluded entirely as they were not hydrolysed by any extract. Phytic acid and phosphoprotein stand out in marked contrast with the other compounds. Phytic acid is attacked readily only by the enzyme in bran extract. Phosphoprotein is the only compound which is hydrolysed by the pancreas. The constitution of phosphoprotein is different from that of the other compounds; it is a protein and is hydrolysed by the proteoclastic enzymes and it seems most likely that the separation of the phosphoric acid is effected by the same enzyme which unlinks the amino-acids from one another. The other organic phosphorus compounds examined are esters of phosphoric acid. The inactivity of the animal tissues most rich in lipase and the activity of an extract of castor oil seeds which does not contain lipase shows that the lipoclastic enzymes have no action on these

esters and that they are hydrolysed by a special group of enzymes. Are we to consider that these esters of phosphoric acid are all hydrolysed by one enzyme, or are we to consider that each compound is hydrolysed by a specific phosphatase? If there were only one phosphatase then phytic acid should be hydrolysed by the enzyme of the intestine, but as this is not the case we must regard phytase as a specific enzyme. The varying extent of the hydrolysis of the other compound by the different extracts supports the supposition that there is a specific enzyme for each phosphoric ester. It can scarcely, however, be admitted that the specificity is really so great. If the chemical constitution of each of the compounds be considered it is seen that glycerophosphoric acid and ethyl phosphoric acid are simple esters, hexose-phosphoric acid is regarded as hexosediphosphoric acid and nucleic acid is a complex made up of hexose or pentose mono-phosphoric acid.

One enzyme is supposed to hydrolyse the group of α-glucosides, another enzyme the group of β-glucosides, invertase separates fructose from sucrose and raffinose, trypsin attacks the polypeptide linking of the amino-acids; consequently it can scarcely be conceived that the specificity of the phosphatases extends to every ester and it would be better to group the phosphatases into several classes such as mono-phosphatases which attack mono-esters like glycerophosphoric acid and ethyl phosphoric acid, di-phosphatases which attack di-esters like hexosephosphoric acid, hexa-phosphatases which attack hexa-esters like phytic acid. Glycerophosphatase will hydrolyse both glycerophosphoric acid and ethyl phosphoric acid as well as other simple esters. Proof of such a supposition will only be given by further work when more simple esters have been examined and when the constitution of hexosephosphoric acid, phytic acid, and nucleic acid have been definitely established.

These experiments confirm the work of Fingerling and Gregersen which shows that the animal organism can synthesise its organic phosphorus compounds from inorganic phosphates. They are not in favour of the belief that organic phosphorus compounds are essential for the well-being of animals, a belief which has arisen from feeding experiments with inorganic and organic phosphorus. No proper control was ever made in such experiments. When phosphoprotein was compared with albumin as the substitute for the supply of protein the nutritive value of albumin was reckoned as equivalent to that of caseinogen, no attention being paid to the different composition of these proteins in amino-acids; when phytic acid, or phytin, was compared with inorganic phosphate the control was not made with inositol and phosphoric acid. Further, if assimilation of organically combined phosphorus occurred,

both phosphoprotein and phytic acid should be found in the blood and tissues. Phosphoprotein occurs only as the secretion of the mammary gland in mammals and of the yolk gland in birds; in these tissues it is synthesised. Phytin has never been found in animals but inositol has been found in most tissues.

The organic phosphorus compounds except phytic acid are all hydrolysed by the intestinal mucosa and their fate in assimilation is like that of all the foodstuffs:—the proteins are known to be completely broken down into amino-acids before they enter the circulation; the fats are hydrolysed as they pass through the intestinal wall; the disaccharides are broken down by enzymes in the mucous membrane of the small intestine. The organic phosphorus compounds are therefore almost certainly assimilated as inorganic phosphate and the organic radicle with which the phosphorus is combined.

The non-hydrolysis of phytic acid by the intestinal mucosa was at first attributed to the experiment being made with carnivora (dog and cat), to which animals vegetable food is not natural, but the same experiment with the herbivora (sheep, ox and rabbit) required some further explanation as to the manner of assimilation of this compound. The excretion of the phosphorus of phytic acid as inorganic phosphate, which was shown by Scofone, Giascosa, Mendel and Underhill and also Horner, pointed to the hydrolysis of the compound. If the absorption be not complete, as the above workers have pointed out, unchanged phytic acid should be present in the faeces. A rabbit was therefore fed for three weeks on food containing a large proportion of bran, and its faeces were examined at the end of this period for organic and inorganic phosphate. The faeces were extracted with dilute hydrochloric acid; inorganic and total P_2O_5 were estimated in the extract by precipitation with ammonium magnesium citrate and by Neumann's method. The phosphorus present in the extract was almost entirely inorganic P_2O_5, as is shown by the following figures:

			Extract		Residue
	H_2O	Total P_2O_5	Total P_2O_5	Inorganic P_2O_5	Total P_2O_5
Nov. 30, 1911	14·8 per cent.	1·9 per cent.	1·5 per cent.	1·4 per cent.	0·2 per cent.
Dec. 1	27·7	1·7	1·6	1·4	—
,, 5	30·0	1·7	1·6	1·5	0·2
,, 6	25·6	2·0	2·0	1·9	—
,, 8	35·3	1·9	1·7	1·4	0·2

Hydrolysis of the phytic acid had occurred; this is not caused by the acid employed in extracting the faeces, for dilute acid at the ordinary temperature does not hydrolyse phytic acid (see following paper). Since the

animal was fed on bran the hydrolysis must be effected by the phytase in the foodstuff. The assimilation of phytic acid by the herbivora thus resembles that of cellulose, which was shown by Horace Brown [1892] to be hydrolysed by the cytase present in the vegetable food.

In carnivora and man, the assimilation of the phosphorus of phytic acid is also in the form of inorganic phosphate. Rogoginski [1910] has shown that in dogs the unabsorbed phytin is present as such in the faeces and that in man the phytic acid is hydrolysed by the bacteria in the large intestine. He also finds no beneficial effect on metabolism from the organic phosphorus compounds. Absorption of phytic acid by animals would require rapid dialysis through the semi-permeable membranes. The dialysis of phytic acid through parchment membranes is slow and is only complete after several days. This is shown by the following experiments:

	26 c.c. sodium phytate inside 50 c.c. water outside P_2O_5 in 5 c.c.		30 c.c. sod. phyt. inside 100 c.c. water outside P_2O_5 in 5 c.c.		70 c.c. sod. phyt. inside 200 c.c. water outside P_2O_5 in 5 c.c.	
	Inside	Outside	Inside	Outside	Inside	Outside
	26·9 mgm.	0 mgm.	31·5 mgm.	0 mgm.	32·5 mgm.	0 mgm.
After 1 day	—	1·5	—	4·4	—	2·7
„ 2 days	—	5·3	—	7·0	—	5·1
„ 3 „	—	—	—	7·6	—	6·7
„ 4 „	11·5	9·3	8·0	8·0	20·0	6·6

In all probability the membrane of the intestine of animals behaves in the same way as the semi-permeable membrane of plant seeds, which does not allow acids to pass through, as was shown by Adrian Brown [1909].

There is thus no evidence of the absorption of phytic acid; it will be hydrolysed either by the enzyme in the foodstuffs, or by bacteria in the large intestine, and will enter the circulation of animals as inositol and phosphoric acid.

The value of the organic phosphorus compounds in nutrition entirely depends on the nature of the organic matter with which the phosphoric acid is combined.

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V. THE HYDROLYSIS OF ORGANIC PHOSPHORUS COMPOUNDS BY DILUTE ACID AND BY DILUTE ALKALI.

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(*Received November 23rd, 1912.*)

Plimmer and Scott [1908] have shown that phosphoproteins may be distinguished from other organic phosphorus compounds occurring in animal tissues by their behaviour with dilute caustic soda. The phosphorus of the phosphoproteins is completely separated as inorganic phosphate by one per cent. caustic soda at 37° C. in twenty-four hours. Dilute caustic soda at 37° C. does not decompose the other natural organic phosphorus compounds but their hydrolysis at higher temperatures has never been investigated. Neumann's method of preparation of nucleic acid, in which the tissues are heated with caustic alkali, shows that nucleic acid in comparison with phosphoprotein is much more stable; it is not definitely known if the nucleic acid is hydrolysed by alkali. Hexosephosphoric acid is readily decomposed by alkali. The synthetical esters of phosphoric acid with methyl, ethyl and other alcohols are stable to alkali, as was shown by Lossen and Köhler in 1891.

In order to determine the composition of the natural organic phosphorus compounds they are hydrolysed by heating with acid at a high temperature and very frequently under pressure. The hydrolysis of glycerophosphoric acid by acids has recently been investigated by Malengreau and Prigent [1911] who find that the decomposition proceeds according to the laws of a monomolecular reaction and that it corresponds to the hydrolysis of ethyl phosphoric acid and other esters of phosphoric acid studied by Cavalier [1899].

There appear to be no further data concerning the hydrolysis of the natural organic phosphorus compounds by acids and alkalies. The statement is usually made that they are decomposed very easily and that loss, due to hydrolysis, occurs in their isolation from tissues when the solutions are being concentrated. More definite information as to their hydrolysis in solution,

both alone and in the presence of acid and alkali, is therefore required. Such data are not only of use in their preparation, but may also serve for the elucidation of their constitution. More practical knowledge concerning the hydrolysis of phytic acid by acids was required for the estimation of inorganic phosphoric acid in the presence of phytic acid and for the comparison of the hydrolysis of the various compounds with the hydrolysis by enzymes (see preceding paper). The experiments have been carried out for these purposes and no attempt has been made to ascertain the kinetics of the reaction.

Experimental.

Dilute acid and dilute alkali of concentrations varying from normal to twice normal have been used. The hydrolysis by acids was carried out in glass vessels, that by alkali in copper vessels. Porcelain vessels were as unsuitable as glass owing to the solvent action of the alkali upon the glaze. The copper vessels were also attacked by the alkali: the solution generally contained dissolved copper as shown by the blue-green colour; the copper compound was precipitated on neutralising with acid and a colourless filtrate was obtained. The solution of the organic phosphorus compound was either mixed with the acid or alkali and samples of equal volume were placed in separate vessels which were maintained at the desired temperature, or the entire mixture was placed in a thermostat. The vessel was then removed, cooled to room temperature and samples taken for analysis.

The same preparations of glycerophosphoric acid, phytic acid, ethyl phosphoric acid and the same trypsin digest were used as described in the previous paper: hexosephosphoric acid was again kindly placed at my disposal by Dr Young: the nucleic acid was again prepared for me by Miss Skelton by Kossel's method and Mr Page gave me a further quantity of hydroxymethyl-phosphinic acid.

The analyses of phosphoric acid were made by precipitation with ammonium magnesium citrate and conversion into magnesium pyrophosphate. Total phosphoric acid in the solution was estimated in a separate sample by Neumann's method. All results were then calculated out in terms of P_2O_5 for the same volume.

As mentioned in the previous paper, inorganic P_2O_5 is not precipitated in the presence of phytic acid. Inorganic P_2O_5 was therefore estimated by precipitation with ammonium molybdate. The hydrolysis by acids was carried out with nitric acid. In most experiments the phytin was dissolved in the nitric acid; there was no necessity to remove the calcium as its presence in the solution does not interfere with the precipitation of the

ammonium phosphomolybdate. When alkali was used the cooled sample was neutralised with nitric acid, an equal volume of 2N nitric acid, and then 20–30 c.c. of 10 per cent. ammonium molybdate solution added; the precipitation began almost immediately and in 24 hours the solution was quite colourless. The precipitate was then filtered off and estimated by the Neumann method by solution in seminormal caustic soda and titration with seminormal sulphuric acid after boiling off the ammonia. The analytical figures obtained are evidence that inorganic P_2O_5 can be estimated by this method in the presence of phytic acid. Details concerning the inhibition of the precipitation of inorganic P_2O_5 by ammonium magnesium citrate in presence of phytic acid and the estimation by ammonium molybdate at room temperature will be given in a later paper dealing with the analysis of phytin.

The details of these experiments are:

I. *Hydrolysis of Glycerophosphoric Acid.*

(*a*) *By Acid.*

i. By $\frac{N}{4}$ H_2SO_4 at 86° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 86° C.

	P_2O_5 in gm.
At commencement	0·0069
After 3 hours ...	0·0092
,, 6 ,, ...	0·0112
,, 15 ,, ...	0·0391
,, 24 ,, ...	0·0494
,, 2 days ...	0·0756
,, 3 ,, ...	0·0855
,, 6 ,, ...	0·0906
Total	0·2675

ii. By $\frac{N}{4}$ H_2SO_4 at 92° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. with water. 50 c.c. samples + 50 c.c. $\frac{N}{2}$ H_2SO_4 kept at 92° C.

	P_2O_5 in gm.
At commencement	0·0102
After 1 day ...	0·0547
,, 2 days ...	0·0683
,, 3 ,, ...	0·0928
,, 4 ,, ...	0·0998
,, 6 ,, ...	0·1578
,, 10 ,, ...	0·2629
Total	0·2587

(*b*) *Autohydrolysis.*

i. At 95° C.

20 c.c. glycerophosphoric acid diluted to 500 c.c. 50 c.c. samples kept at 95° C.

	P_2O_5 in gm.
At commencement	0·0074
After 2 hours ...	0·0114
,, 4 ,, ...	0·0154
,, 6 ,, ...	0·0179
,, 8 ,, ...	0·0194
,, 16 ,, ...	0·0342
Total	0·2612

ii. At 75° C.

25 c.c. glycerophosphoric acid diluted to 500 c.c. and kept at 75° C.

	P_2O_5 in gm.
At commencement	0·0157
After 2 days ...	0·0216
,, 3 ,, ...	0·0281
,, 4 ,, ...	0·0341
,, 5 ,, ...	0·0404
,, 6 ,, ...	0·0458
,, 7 ,, ...	0·0512
,, 9 ,, ...	0·0623
Total	0·1374

(*c*) *By Alkali.*

i. By $\frac{N}{1}$ NaOH at 95° C.

25 c.c. glycerophosphoric acid diluted with 700 c.c. $\frac{N}{1}$ NaOH.

	P_2O_5 in gm.
At commencement	0·0137
After 2 days ...	0·0144
,, 3 ,, ...	0·0118
,, 5 ,, ...	0·0150
,, 7 ,, ...	0·0195
,, 10 ,, ...	0·0179
Total	0·2194

ii. By $\frac{2N}{1}$ NaOH at 75° C.

6 gm. sodium glycerophosphate dissolved in 550 c.c. $\frac{2N}{1}$ NaOH (5 c.c. = 20·1 c.c. $\frac{N}{2}$ H_2SO_4) and kept at 75° C.

	P_2O_5 in gm.
At commencement	——
After 2 days ...	0·0011
,, 4 ,, ...	0·0023
,, 8 ,, ...	0·0010
,, 16 ,, ...	0·0018
,, 32 ,, ...	0·0033
,, 81 ,, ...	0·0078
Total	0·1750

II. *Hydrolysis of Ethyl Phosphoric Acid.*

(*a*) By acid $\left(\frac{N}{1}\text{ HCl}\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ HCl.

	P_2O_5 in gm.
At commencement	0·0001
After 8 hours ...	0·0003
,, 1 day ...	0·0013
,, 2 days ...	0·0031
,, 3 ,, ...	0·0045
,, 4 ,, ...	0·0065
,, 5 ,, ...	0·0077
,, 6 ,, ...	0·0091
,, 8 ,, ...	0·0115
,, 11 ,, ...	0·0146
,, 16 ,, ...	0·0204
,, 20 ,, ...	0·0240
Total	0·0469

(*b*) By alkali $\left(\frac{N}{1}\text{ NaOH}\right)$ at 75° C.

300 c.c. sodium ethyl phosphate solution + 300 c.c. $\frac{2N}{1}$ NaOH.

	P_2O_5 in gm.
At commencement	0
After 1 day ...	0
,, 5 days ...	0
,, 13 ,, ...	0
,, 33 ,, ...	0
Total	0·0926

(*c*) Autohydrolysis at 75° C. 200 c.c. ethyl phosphoric acid solution.

	P_2O_5 in gm.
At commencement	0·0002
After 1 day ...	0·0018
,, 2 days ...	0·0033
,, 4 ,, ...	0·0064
,, 6 ,, ...	0·0094
,, 50 ,, ...	0·0526
Total	0·0746

III. *Hydrolysis of Phytic Acid.*

(*a*) *By Acid.*

i. By $\frac{N}{1}$ HNO_3 at 37° C.

125 c.c. phytic acid solution + 125 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0127
After 1 day ...	0·0144
,, 2 days ...	0·0142
,, 5 ,, ...	0·0134
Total	0·2156

ii. By $\frac{2N}{1}$ HNO_3 at 37° C.

1 gm. phytin dissolved in 250 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0055
After 2 days ...	0·0062
,, 5 ,, ...	0·0062
,, 8 ,, ...	0·0066
Total	0·0976

iii. By $\frac{N}{1}$ HNO_3 at 65° C.

1 gm. phytin dissolved in 130 c.c. H_2O + 130 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0051
After 1 hour ...	0·0056
,, 2 hours ...	0·0057
,, 4 ,, ...	0·0054
,, 6 ,, ...	0·0058
Total	0·0938

iv. By $\frac{2N}{1}$ HNO_3 at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0046
After 15 mins. ...	0·0051
,, 30 ,, ...	0·0053
,, 1 hour ...	0·0053
,, 2 hours ...	0·0057
Total	0·0913

v. By $\frac{2N}{1}$ HNO_3 at 64° C.

1 gm. phytin dissolved in 260 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0049
After 3 hours ...	0·0056
,, 6 ,, ..	0·0060
,, 9 ,, ...	0·0061
,, 24 ,, ...	0·0065
Total	0·1040

vi. By $\frac{N}{1}$ HNO_3 at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0063
After 1 day ...	0·0134
,, 2 days ...	0·0232
,, 4 ,, ...	0·0280
,, 8 ,, ...	0·0439
Total	0·0926

vii. By $\frac{N}{1}$ HNO_3 at 75° C.

1 gm. phytin dissolved in 260 c.c. $\frac{N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0056
After 1 day ...	0·0116
,, 2 days ...	0·0165
,, 4 ,, ...	0·0259
,, 8 ,, ...	0·0436
Total	0·1014

viii. By $\frac{2N}{1}$ HNO_3 at 75° C.

2·5 gm. phytin dissolved in 500 c.c. $\frac{2N}{1}$ HNO_3.

	P_2O_5 in gm.
At commencement	0·0077
After 1 day ...	0·0216
,, 2 days ...	0·0349
,, 4 ,, ...	0·0571
,, 6 ,, ...	0·0777
,, 8 ,, ...	0·0976
,, 9 ,, ...	0·1040
,, 11 ,, ...	0·1147
,, 12 ,, ...	0·1192
,, 14 ,, ...	0·1243
,, 15 ,, ...	0·1255
,, 16 ,, ...	0·1274
,, 17 ,, ...	0·1312
Total	0·1471

(*b*) *By Alkali.*

By $\frac{N}{1}$ NaOH at 75° C.

300 c.c. sodium phytate solution + 300 c.c. $\frac{2N}{1}$ NaOH. (5 c.c. = 9·7 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0·0093
After 1 day ...	0·0089
,, 2 days ...	0·0091
,, 4 ,, ...	0·0079
,, 8 ,, ...	0·0094
,, 16 ,, ...	0·0106
,, 32 ,, ...	0·0119
Total	0·1407

(*c*) *Autohydrolysis at* 75° C.

600 c.c. phytic acid solution.

	P_2O_5 in gm.
At commencement	0·0034
After 1 day ...	0·0071
,, 2 days ...	0·0112
,, 3 ,, ...	0·0151
,, 4 ,, ...	0·0191
,, 5 ,, ...	0·0235
,, 7 ,, ...	0·0314
Total	0·0527

IV. *Hydrolysis of Hexosephosphoric Acid.*

(*a*) By acid $\left(\frac{N}{1}\text{ HCl}\right)$ at 75° C.

100 c.c. hexosephosphoric acid solution neutralised with NaOH + 100 c.c. $\frac{2N}{1}$ HCl. (5 c.c. = 11·2 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0034
After 7 hours ...	0·0348
,, 1 day ...	0·0443
,, 2 days ...	0·0480
,, 3 ,, ...	0·0486
,, 5 ,, ...	0·0508
,, 8 ,, ...	0·0508
Total	0·0527

(*b*) By alkali $\left(\frac{1·5N}{1}\text{ NaOH}\right)$ at 75° C.

40 c.c. hexosephosphoric acid solution + 500 c.c. $\frac{2N}{1}$ NaOH. (5 c.c. = 16·5 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0·0136
After 1 day ...	0·1610
,, 2 days ...	0·1598
,, 4 ,, ...	0·1641
Total	0·1978

(*c*) Autohydrolysis at 75° C.

50 c.c. hexosephosphoric acid solution + 220 c.c. water. (5 c.c. = 0·2 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0037
After 1 day ...	0·0494
,, 2 days ...	0·0698
,, 3 ,, ...	0·0791
,, 18 ,, ...	0·1152
Total	0·1243

V. *Hydrolysis of Nucleic Acid.*

(*a*) By acid $\left(\frac{N}{1}\text{ HCl}\right)$ at 75° C.

3·5 gm. thymus nucleic acid dissolved in 200 c.c. H_2O + 10 c.c. $\frac{2N}{1}$ NaOH; 210 c.c. $\frac{2N}{1}$ HCl then added; the precipitate went into solution in 15 minutes at 75° C.

	P_2O_5 in gm.
At commencement	——
After 4 hours ...	0·0250
,, 1 day ...	0·0429
,, 2 days ...	0·0545
,, 3 ,, ...	0·0612
,, 4 ,, ...	0·0968
,, 8 ,, ...	0·1106
Total	0·1103

(*b*) By alkali $\left(\frac{N}{1}\text{ NaOH}\right)$ at 75° C.

6 gm. nucleic acid dissolved in 600 c.c. $\frac{N}{1}$ NaOH. (5 c.c. = 10 c.c. $\frac{N}{2}$ H_2SO_4.)

	P_2O_5 in gm.
At commencement	0
After 1 day ...	0·0040
,, 2 days ...	0·0106
,, 4 ,, ...	0·0179
,, 16 ,, ...	0·0369
,, 32 ,, ...	0·0510
,, 76 ,, ...	0·0990
Total	0·1128

(*c*) By alkali $\left(\frac{N}{1}\text{ NaOH}\right)$ at 70° C.

4 gm. nucleic acid dissolved in 200 c.c. H_2O + 200 c.c. 2N NaOH.

	P_2O_5 in gm.
At commencement	0·0020
After 1 day	0·0108
,, 2 days	0·0145
,, 4 ,, ...	0·0236
,, 9 ,, ...	0·0305
,, 22 ,, ...	0·0447
Total	0·0951

VI. *Hydrolysis of Phosphoprotein by Alkali.*

i. 200 c.c. trypsin digest of caseinogen + 50 c.c. $\frac{2N}{1}$ NaOH. (=1·6 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0194
After 1 day ...	0·0320
,, 2 days ...	0·0321
,, 3 ,, ...	0·0328
Total	0·0342

ii. 175 c.c. trypsin digest of caseinogen + 25 c.c. $\frac{2N}{1}$ NaOH. (=1 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0199
After 1 day ...	0·0327
,, 2 days ...	0·0342
Total	0·0380

iii. 180 c.c. trypsin digest of caseinogen + 20 c.c. $\frac{2N}{1}$ NaOH. (=0·8 per cent. NaOH.)

	P_2O_5 in gm.
At commencement	0·0209
After 1 day ...	0·0272
,, 2 days ...	0·0300
,, 4 ,, ...	0·0334
Total	0·0406

VII. *Hydrolysis of Hydroxymethyl-phosphinic Acid by Acid.*

130 c.c. sodium hydroxymethyl-phosphinate solution + 130 c.c. $\frac{2N}{1}$ HNO_3. (5 c.c. = 10·3 c.c. $\frac{N}{2}$ NaOH.)

	P_2O_5 in gm.
At commencement	0·0006
After 1 day	0·0004
,, 2 days	0·0006
,, 4 ,, ...	0·0006
,, 8 ,, ...	0·0018
Total	0·1179

Glycerophosphoric acid is slowly hydrolysed by dilute acid, complete separation of the phosphoric acid requiring 10 days at 92° C. The glycerol, which is formed, was isolated in another experiment by removing the sulphuric and phosphoric acids by baryta, concentrating and distilling *in vacuo*: the glycerol distilled at 162° C. at 10–15 mm. pressure and a yield of 44 per cent. was obtained. Autohydrolysis is considerably slower; one-eighth of the glycerophosphoric acid was decomposed in 16 hours at 95° C., and only one-half in 9 days at 75° C. It is not hydrolysed by alkali; the slight increase in the amount of inorganic phosphate observed after 81 days by the action of twice normal caustic soda was due to slight evaporation through the cork; the total P_2O_5 at the end of the period being 0·1750 gm., which is a little greater than that at the beginning—0·1509 gm.

Glycerophosphoric acid is stable to alkali like ethyl phosphoric acid; Lossen and Köhler's result has been confirmed and autohydrolysis and acid hydrolysis of ethyl phosphoric acid have been carried out at 75° C. for comparison with the other compounds. Autohydrolysis of ethyl phosphoric acid was not complete in 50 days and the hydrolysis by acid seems to be slower than in the case of glycerophosphoric acid.

Phytic acid is the most stable of the organic phosphorus compounds. At 37° C. dilute acid effected no hydrolysis; at 64° C. a slight hydrolysis could be detected in 24 hours; at 75° C. normal nitric acid separated about half of the phosphorus as inorganic phosphate in 8 days. Complete hydrolysis was not effected by twice normal nitric acid in 17 days at the same temperature. Autohydrolysis at 75° C. resulted in the splitting off of about half the phosphoric acid in 7 days.

Phytic acid is also quite stable to alkali; the slight increase in the amount of inorganic phosphate noted after 32 days by normal alkali at 75° C. is due to concentration of the solution by evaporation through the cork.

Hexosephosphoric acid is easily hydrolysed by both acid and alkali. Complete hydrolysis was effected by normal hydrochloric acid in 3–5 days, the greater part of the phosphoric acid being separated in 1 day. Autohydrolysis at 75°C. was much slower than acid hydrolysis; half the phosphoric acid was separated in 2 days; complete hydrolysis occurred in about 18 days. Dilute alkali produced complete hydrolysis in 1 day. In all the experiments a small quantity of organic phosphorus remained undecomposed: it is very probable that another organic phosphorus compound was present as impurity in the solution.

Nucleic acid is hydrolysed by both acid and alkali. Normal hydrochloric acid effected complete hydrolysis in 8 days at 75° C. With normal caustic soda a slow hydrolysis was observed; about one-third of the phosphoric acid was split off in 8 days, about one-half in 32 days; even after 76 days a small amount of organic phosphorus remained in solution.

The organic phosphorus compound which remains in a prolonged tryptic digest of caseinogen is also completely hydrolysed by 1 per cent. caustic soda; there is no difference in its behaviour from that of caseinogen; the former observation of Plimmer and Bayliss [1906] was incorrect.

Hydroxymethyl-phosphinic acid is not hydrolysed by dilute acid at 75° C.

Summary and Conclusion.

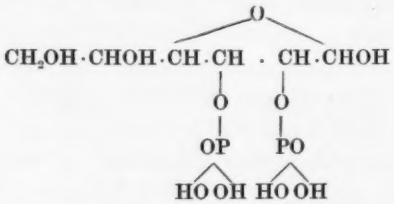
Ethyl phosphoric acid, glycerophosphoric acid and phytic acid are hydrolysed by acid, but are stable to alkali. Stability to alkali is therefore a property of the esters of phosphoric acid.

Hexosephosphoric acid and phosphoprotein are so different in their behaviour to alkali from the above three compounds that some difference in their constitution from that of the esters must exist.

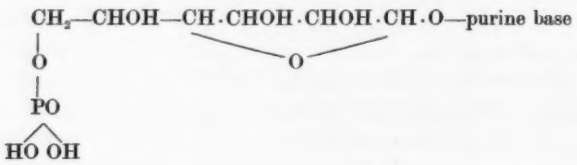
It is not known how the phosphoric acid is combined in phosphoprotein, but it is probably united with one of the amino-acids.

Hexosephosphoric acid reduces Fehling's solution which points to the presence of the functional aldehyde or ketone group in the molecule.

The phosphoric acid radicles are most probably combined with two of the hydroxyl groups leaving the reducing group free. The action of the alkali will destroy this grouping and the whole carbohydrate molecule will be decomposed leaving the phosphoric acid:



Nucleic acid, since it is hydrolysed like hexosephosphoric acid by both acid and alkali, seems to occupy an intermediate position between the stable esters and the very unstable hexosephosphoric acid. If the purine, or pyrimidine, base be attached to the functional aldehyde group in the same way as the alcohols in the glucosides, the action of alkali may be to destroy the purine base leaving the aldehyde group for decomposition of the molecule, and phosphoric acid will remain. A formula such as



would explain the slow decomposition by alkali.

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VI. THE NITROGENOUS CONSTITUENTS OF LIME-JUICE.

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(*Preliminary Communication.*)

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(*Received November 22nd, 1912.*)

In connection with the work previously published on beri-beri [Funk, 1911, 1912, 1], in which the physiological importance of certain nitrogenous substances, belonging most probably to the pyrimidine group, was demonstrated, a similar fractionation of lime-juice was undertaken for the investigation of scurvy.

From the classical work of Holst and Fröhlich [1907, 1912] on scurvy we know that this disease has a close analogy to beri-beri and is also due to a lack of some substance in the food. They specially advanced our knowledge of this disease by inducing in animals (rabbits and guinea-pigs) a disease which possesses a great similarity in symptoms to human scurvy. They produced the experimental scurvy by an exclusive diet of oats or autoclaved vegetables and were able to prevent it by an addition to this diet of fresh vegetable-juice, unboiled milk or lime-juice. Lime-juice itself has been known for many years as an excellent remedy for scurvy.

Some time ago I was able to show [1912, 2] that lime-juice contains an anti-neuritic substance curing polyneuritis in birds, produced by an exclusive diet of polished rice, and a substance, present in another fraction, which is only capable of prolonging the life of these birds. Further experiments have shown that pyrimidine substances in general possess this life-prolonging action and a paper on this subject will shortly appear. No anti-scorbutic substance however was obtained from lime-juice.

I had at first intended to try each fraction, obtained during this

investigation, on guinea-pigs fed on oats, but in this I found considerable difficulties. The animals refused all food after a period of three weeks and even an addition of freshly made potato-juice or lime-juice was not able to save them from starvation. The same negative result was obtained with fractions of lime-juice which might have been expected to contain the anti-scorbutic substance in a more concentrated form.

The only positive results were obtained with milk, 50 c.c. daily of unboiled milk in addition to the oats diet preventing loss of weight and onset of scurvy. Even after the elimination of caseinogen and the other proteins present in milk, the latter keeps its preventive power. In doing this extreme care must be taken not to destroy the substance, and all boiling must be carefully avoided. The caseinogen is precipitated by addition of acetic acid at 50° and lactalbumin by dialysed iron-solution, kaolin however carries the substance down. The solution is then concentrated by freezing it and taking the supernatant liquid. This subject is being further investigated. This unstable character of the anti-scorbutic substance renders it unlikely that the usual method of separation, with the unavoidable use of alkali, would lead to its isolation. Although no anti-scorbutic substance was obtained from lime-juice, several new compounds have been detected and are described below.

Besides a substance, which apparently belongs to the terpene group and which has only been analysed and not further investigated, as having no direct connection with the subject, three nitrogenous substances have been isolated. One belongs to the purine group, the second to the pyrimidine group and the third to the choline group. The small yield obtained did not allow however of a complete investigation, so that this paper must be regarded as preliminary.

Experimental.

407 litres of commercial lime-juice were precipitated in portions of 15 litres each with 2800 grms. of neutral lead acetate. The bulky precipitate was filtered off on a large Buchner funnel and the residue pressed out in a hydraulic press. The combined filtrates were freed from the excess of lead, first by an addition of sulphuric acid, then by sulphuretted hydrogen, and were then concentrated *in vacuo* at low temperature. During the distillation crystals separated out, which were filtered off, and weighed when dry 258 grms. These were recrystallised from 20 % acetic acid and 162 grms. were obtained in needles. The substance proved to be free from nitrogen and was recrystallised for analysis once more from water. It is

nearly insoluble in all other solvents, except acetic acid, and is volatile with steam; M.P. 97°–100° (uncorr.).

0·1357 grm. gave	0·3400 CO_2 and	0·1312 H_2O.
Found	68·33 % C;	10·73 % H.
Calc. for $C_{13}H_{24}O_3$;	68·42 % C;	10·52 % H.

This substance probably belongs to the terpene group; it possesses a very characteristic agreeable smell when heated. The nature of this compound, which has no direct relationship with the scurvy problem, was not further investigated.

To the filtrate from this substance enough sulphuric acid was added to make a 5 % solution and then a 50 % solution of phospho-tungstic acid until precipitation was complete. The precipitate obtained amounted to nearly 12 kg., and consisted chiefly of potassium salt.

Investigation of the Phospho-tungstate Precipitate.

The precipitate was decomposed in portions of 1 kg. each with 2½ kg. baryta in a mortar and the mixture was shaken on the shaking machine for one hour. The precipitate was then filtered off, suspended in water and again shaken. The excess of baryta was eliminated from the combined filtrates by the careful addition of dilute sulphuric acid. The solution obtained was then neutralised with nitric acid, as it was strongly alkaline, and evaporated *in vacuo* to about two litres. In this solution the usual fractionation with silver nitrate was performed and three fractions were obtained. The first fraction came down on addition of a silver nitrate solution and contained the substances of the purine group. This precipitate was filtered off and to the filtrate a saturated solution of silver nitrate was added until a drop of the liquid gave with a cold baryta solution a brown precipitate which indicates an excess of silver. A saturated aqueous baryta solution was then added until a drop of the clear liquid gave only a small precipitate with silver nitrate and ammonia. The second precipitate consists of pyrimidine bases and substances of the histidine type. To the filtrate pulverised baryta was added until the whole of the silver was precipitated. The fraction thus obtained contains, in addition to traces of pyrimidine substances, compounds of the arginine group. The last filtrate contains substances of the choline group. All the fractions were investigated separately.

Investigation of the first Silver fraction.

The bulky precipitate obtained by a simple addition of silver nitrate was filtered off, well washed with water, suspended in water and the double silver nitrate salts converted into the silver salts by heating them on the water-bath with ammonia. The precipitate was filtered off and washed with water, until the filtrate was free from nitric acid. It was then suspended in water and decomposed with sulphuretted hydrogen. The solution on evaporation yielded 1 grm. of substance, which however contained a little ash. It was recrystallised from water and gave 0·7 grm. of crystals in the form of plates. After drying *in vacuo* at 110° they became brown at 240° and melted at 282° (corr.). The substance is precipitated by mercuric acetate and partially by gold chloride. It differs from the known purine bodies by the great solubility in hot water and the absence of all known reactions for these derivatives. Heated with nitric acid it becomes yellow, and brown when more strongly heated, no change of colouration being produced on the addition of alkali.

0·3855 grm. loses at 110° (*vacuo*) 0·0190 grm. H_2O
0·1176 ,, requires 32·7 c.c. N/10 H_2SO_4
0·1372 ,, ,, 38 c.c. ,, ,,
0·1226 ,, gave 0·1788 CO_2 and 0·0196 H_2O

Calc. for $C_6H_7O_2N_5$ (Mol. wt. = 181)	Found	
C 39·78 %	39·77	
H 3·86 %	3·55	
N 38·67 %	38·77	38·92
½ Mol. H_2O 4·73 %	4·92	

Investigation of the second Silver fraction.

The second silver nitrate precipitate was filtered off, washed well with water and decomposed with sulphuretted hydrogen. In the filtrate the last traces of baryta were taken out with a very dilute solution of sulphuric acid and the solution was evaporated *in vacuo*. As the residue did not deposit any crystals, the solution was precipitated with mercuric sulphate. The solution obtained by decomposition of the precipitate showed no tendency to crystallisation, but on addition of a picric acid solution an oil separated, which was decanted and dissolved in acetone. After evaporation a solid powder was obtained which amounted to 0·35 grm. This was recrystallised from a mixture of alcohol and acetone and yielded about 0·1 grm. of brown prisms, which decomposed at 205°–210°. An amount of this fraction

corresponding to 0·01 grm. of the picrate was decomposed and given to a polyneuritic pigeon which showed a considerable improvement and died only after four days.

The mercuric sulphate filtrate, freed from mercury and sulphuric acid, was evaporated *in vacuo* and the residue left in a desiccator after addition of alcohol. After a few days a colourless substance separated out which amounted to 0·7 grm. After recrystallisation from dilute alcohol 0·25 grm. was obtained in the form of microscopical spherolites, which after drying at 110° *in vacuo*, melted and decomposed at 188°–189°.

0·0958 grm. subst. required 7·5 c.c. N/10 H_2SO_4; 10·96 % N.
0·1034 „ gave 0·1625 CO_2 and 0·0636 H_2O; 42·86 % C; 6·83 % H.
Calc. for $C_9H_{18}O_6N_2$ (Mol. wt. = 250) 43·2 % C; 7·2 % H and 11·2 % N.

The third silver fraction yielded but little substance. On evaporation of the solution obtained by decomposing the precipitate no crystalline compound separated out. Picric acid solution however gave a precipitate which after standing formed crystalline, yellow spherolites, which began to decompose at 260°; yield 0·1 gr.

Investigation of the Silver Nitrate Filtrate.

Of this filtrate, which amounted to 4600 c.c., only 220 c.c. were taken for further investigation. To this liquid after elimination of silver and baryta, phospho-tungstic acid was added and 420 grms. of a dry precipitate were obtained, which was decomposed in the ordinary way. The solution of the bases belonging to the choline group was neutralised with hydrochloric acid and evaporated *in vacuo* to dryness. To get rid of the last traces of water the residue was redissolved in alcohol and the alcohol evaporated *in vacuo*. Finally an alcoholic solution was made and an alcoholic solution of sublimate was added as long as a precipitate was formed. This was recrystallised from hot water with the addition of a little sublimate. It was found to be very slightly soluble in water. In this way 10·5 grms. of crystals were obtained in the form of cubes. These were redissolved in water and decomposed with sulphuretted hydrogen. The filtrate was evaporated *in vacuo* to dryness, the residue dissolved in alcohol and evaporated again. The alcoholic solution finally obtained was precipitated with an alcoholic platinic chloride solution, 1·3 grms. of a pale yellow precipitate being obtained which was recrystallised from dilute alcohol; 0·85 grm. was thus obtained in the form of needles, which, after drying at 110° *in vacuo*, melted at 220° (uncorr.). The substance gave the following figures on analysis, after being dried *in vacuo* at 110°.

0·1666 grm. gave 0·0451 grm. Pt
0·1622 „ required 4·2 c.c. N/10 H_2SO_4
0·2173 „ gave 0·2104 CO_2; 0·0878 H_2O; 0·0589 Pt and 0·2586 AgCl.

Calc. for $(C_5H_{12}O_2NHCl)_2PtCl_4$ (Mol. wt. 724).	Found:
C: 26·52;	26·40
H: 4·42;	4·48
N: 3·86	3·62
Pt: 26·94;	27·07; 27·1
Cl: 29·55	29·44

On igniting the platinum salt a peculiar smell was noticed, which was quite different from that given by choline.

A substance of this formula, with similar properties (M.P. 219°), was described by v. Braun [1908] as the platinichloride of methylpiperidylacetic betaine. The substance is being further investigated.

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VII. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. I. METHOD OF PREPARATION.

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(*Received November 23rd, 1912.*)

I have previously [Wheldale, 1909, 2, 3; 1910, 2; 1911] made certain suggestions as to the nature of the chemical reactions involved in the formation of anthocyanin. There is little doubt that anthocyanin, as a collective term, in the same sense as protein, sugar, tannin, etc., includes many substances having in general similar properties, but differing among themselves as regards constitution. Evidence, from various sources, has led me to conclude that some anthocyanins may be derived from members of the groups of natural yellow colouring matters, known as the flavones and xanthones.

The yellow colouring matters are largely present in the plants as glucosides, some, or possibly all the hydroxyl groups, being replaced by sugar. I have suggested that the reactions involved in the formation of anthocyanin may be represented, in very general terms, as follows:—

$$\text{Glucoside} + \text{Water} \rightleftarrows \underset{\text{(Flavone or Xanthone)}}{\text{Chromogen}} + \text{Sugar}$$

$$x\,(\text{Chromogen}) + \text{Oxygen} \longrightarrow \text{Anthocyanin}.$$

The first reaction may be regarded as controlled by one or more glucoside-splitting enzymes and it is conceivable that specific enzymes may act on hydroxyl groups in different positions. When certain hydroxyl groups (position to be determined) are free from sugar, oxidation may take place at these points, or possibly condensation, or both, with the formation of anthocyanin. The residual hydroxyl groups in the anthocyanin molecule would probably be replaced by sugar and hence the anthocyanins would occur as glucosides. There is evidence that the second reaction may be brought about by an oxidase system [Wheldale, 1909, 3; 1910, 2; 1911; Keeble and Armstrong, 1912].

Some of the evidence for the above hypothesis is derived from results obtained in cross-breeding with varieties of *Antirrhinum majus* [Wheldale, 1907; 1909, 1; 1910, 1]. The wild type of this plant has magenta flowers, the colour due to anthocyanin. Among other varieties produced on cultivation are three without anthocyanin, i.e. ivory, yellow and white. Ivory and yellow contain pigments very probably of the flavone class. The white has no such pigment but carries other factors which apparently act on the yellow pigments to form anthocyanin. None of the three varieties alone can produce anthocyanin, but when ivory is crossed with white, a plant having magenta flowers is produced; when yellow with white, a plant having crimson (possibly a mixture of magenta and yellow) flowers.

With a view to testing this hypothesis I commenced a chemical investigation of the pigments of *Antirrhinum* and in the summer of 1911 I succeeded in finding a satisfactory method for obtaining the pigment in quantity in the solid state. Extractions were made from yellow and ivory separately, but all varieties containing magenta anthocyanin (i.e. ivory tinged with magenta, magenta and crimson) were extracted together. In this way all extracts contained mixtures of pigments, as will be seen below on referring to the varieties in detail. When attempting to purify the crude pigment later, I found difficulty in separating some of the pigments. Hence, when I again prepared pigment this year, the precaution was taken, in the case of certain varieties, of extracting portions only of the corolla, containing, to the best of my belief, either one pigment or two, which could be easily separated.

The method of preparation is as follows:—the flowers, picked off the spikes, are boiled with water in saucepans. The pigments are readily soluble in water and the extract is filtered through large funnels into lixiviating jars. The pigments are then precipitated as insoluble lead salts by adding solid crystalline lead acetate until no further precipitate appears. After standing a few hours the greater part of the liquid is decanted from the precipitates, which are then filtered through a large Buchner funnel attached to a filter pump. The lead salts are stirred up with 5–10 % sulphuric acid which decomposes the salts with formation of lead sulphate. The lead sulphate is filtered off and the filtrate contains the pigments as glucosides in dilute sulphuric acid solution. These solutions are boiled for several hours in two-litre Jena flasks fitted with simple tube condensers, care being taken to avoid concentration of the solution, since under such circumstances the pigment may become charred. Hydrolysis of the glucosides takes place during boiling and, on cooling and standing about 12 hours, the pigment, which is less soluble than the glucoside, is deposited. The deposit is separated by

filtering through as small a Buchner funnel as possible, a Geryk pump being used. After washing, the pigment is dried in a desiccator over sulphuric acid.

During the current year (1912), the following varieties were extracted. Excellent coloured figures of the varieties are given by Baur [1911]. Figures below refer to Baur's plate.

Ivory. (Fig. 3.) The corolla contains a pale yellow pigment (ivory) and a patch of deeper yellow pigment (yellow) on the lower lip. Extracts from the whole flower or from the lower lip gave an orange-yellow lead precipitate. From the separated upper lip, a canary-yellow lead precipitate. Pigment was prepared separately from both upper and lower lips.

Yellow. (Fig. 2.) The lips of the corolla contain yellow pigment which is chiefly confined to the epidermal cells. The inner tissues of the lips and the entire tube contain ivory pigment. Extracts gave an orange lead precipitate. Pigment was prepared separately from both upper and lower lips.

Ivory tinged with magenta. (Fig. 6.) Flower, ivory, with some development of magenta anthocyanin which is chiefly confined to the epidermis. Extracts gave a yellow-green lead precipitate. Pigment was prepared from entire flowers only and presumably contains ivory, yellow and magenta.

Magenta. (Fig. 7.) Magenta chiefly confined to epidermis; inner tissues contain ivory and there is a yellow patch on the lower lip. Extracts gave a deep green lead precipitate, some pigment apparently remaining in solution in the acetate, forming a deep green solution which is dichroic, red by transmitted and green by reflected light. Pigment was prepared separately from both upper and lower lips. The upper presumably contains magenta and ivory, the lower, yellow in addition.

Crimson. (Fig. 8.) Lips of the corolla are crimson and the tube magenta. It is at present uncertain whether the crimson colour is due to the presence of both yellow and magenta in the cells or to a distinct crimson pigment. Pigment was prepared from entire flowers only.

Rose doré. (Fig. 15.) Corolla contains a "red" anthocyanin [Wheldale 1909, 2]. The inner tissues, ivory, and there is a yellow patch on the lower lip. Extracts gave a reddish-green lead precipitate. Pigment was prepared from entire flowers only.

Bronze. (Fig. 17.) Bears the same relation to rose doré as crimson to magenta and it is again uncertain whether the colour is due to a mixture or to a separate pigment. Extracts gave a deep-red lead precipitate. Pigment was prepared from entire flowers only.

Methods of purification were first carried out on pigment obtained in 1911. As mentioned above, tinged ivory, magenta and crimson flowers had

then been extracted together. The crude pigment which presumably contained ivory, yellow and magenta was finely powdered thoroughly dried, placed in a Soxhlet thimble, and extracted first with warm ether in which the ivory and yellow pigments are soluble, though not readily. The anthocyanins, both magenta and red, are insoluble in ether.

From the ether extract two pigments were obtained by fractional crystallisation from alcohol and ethyl acetate. The less soluble pigment was taken to be ivory, the more soluble to be yellow. It is doubtful whether either pigment was obtained in the pure state.

Ivory pigment. This is readily soluble in alcohol and acetic acid, with more difficulty in ether and ethyl acetate, very slightly soluble in cold, more so in hot water; insoluble in chloroform and benzene. It crystallises from dilute alcohol in plates; M.P. 338°. It underwent combustion with difficulty in oxygen and did not give constant results for carbon. Tested by Zeisel's method for a methoxyl group, some silver iodide was obtained but only in such quantity as to indicate impurity. An acetyl derivative was prepared by boiling with acetic anhydride and pouring the product into sodium acetate solution. The product was purified with difficulty by crystallisation from ethyl acetate. The final product was pure white and crystallised in glistening needles; M.P. 182°. The combustion results were as follows:

C = 63·19 %	H = 4·30 %
63·21	4·22
62·95	4·31

Of the known flavones, the ivory pigment bears most resemblance to apigenin in properties and acetyl derivative. Opinion is reserved as to its identity until further analyses have been made, especially with the ivory pigment (free from yellow) obtained from the upper lip of the ivory variety.

Yellow pigment. Crystallises in plates from dilute alcohol but was not obtained in the pure state; M.P. 290°–300°.

Magenta pigment. After extraction with ether for several weeks, the magenta pigment was obtained free from yellow. It crystallises, but not well, from a mixture of alcohol and ethyl acetate. It decomposes without melting when heated to 340°.

Other results obtained with magenta and yellow are reserved until further work has been done.

The crude pigment was extracted in the laboratory of the John Innes Horticultural Institution, Merton Park, Surrey. Large numbers of plants, the offspring of varieties originally used in breeding experiments, were grown

in the garden of the Institution. The yield of pigment was small and was not ascertained quantitatively because of the labour involved in weighing the flowers.

The work of purification and analysis, as far as it has gone, was carried out in the Biochemical Laboratory of the Institute of Physiology, University College, London. I am much indebted to Dr Aders Plimmer for kind help and suggestions.

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VIII. THE DENSITY AND SOLUTION VOLUME OF SOME PROTEINS.

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(*Received November 25th, 1912.*)

In a previous communication dealing with the viscosity and other properties of caseinogen solutions [1912], we pointed out that there was a shrinkage in volume and corresponding increase in density when that protein formed colloidal solution. In order to investigate the point the method employed was to determine the density of the dry, powdered protein by weighing in benzene or some other indifferent fluid and to compare the value with that obtained by calculation from the observed density of a fairly concentrated solution. The latter value was found to be much greater than the former.

As far as we have been able to ascertain, this is the first attempt to determine the density of proteins with the exception of gelatin. Quincke [1903] found the specific gravity of a specimen of the latter to be 1·368, while Lüdekings [1888] had previously given the value of 1·412, and had shown that the density calculated from that of jellies (14 to 35 % gelatin) was as high as 1·9.

The increase in density when proteins are dissolved in water is an instance of the phenomenon constantly associated with colloidal solution of the lyophile or emulsoid character. The intimate association of the protein particles with water leads to a shrinkage in total volume, so that the increase in density is more properly regarded as an attribute of the whole system than of the protein alone. Analogous cases are those of gum-tragacanth and starch. In the case of the latter Rödewald [1897] found the density in the dry state and that calculated from that of a solution to be 1·38 and 1·49 respectively.

In our experiment with caseinogen, a sample of the pure, dry, powdered protein was prepared by acidifying a solution in dilute sodium hydroxide, washing and drying the precipitate, and grinding to a fine powder. A known weight was inserted in a pyknometer bottle, covered with benzene of known

density, and all the air expelled by careful heating *in vacuo*. The bottle was then filled up with benzene in a thermostat at 25° C. and weighed. The weight obtained was compared with that of the bottle filled with benzene at the same temperature, and the density of the powder calculated. The value obtained was 1·318 as the mean of two determinations (see Table I). A solution of caseinogen (7·85 %) in dilute sodium hydroxide gave a density of 1·0240, from which the density of sodium caseinogenate was calculated to be 1·42.

In order to compare this value with that obtained by direct determination, it is necessary to apply a small correction, seeing that the solution consisted of sodium caseinogenate. The ash present composed 4·331 % of the dry weight and, assuming this to be sodium carbonate, 2 % of the dry weight would consist of sodium, and the density, for comparison with that of the powdered caseinogen, should be reduced by that amount. After this correction has been made the caseinogen in solution is still seen to be denser than the solid caseinogen in the proportion of about 1·39 to 1·318.

In addition to caseinogen three other pure proteins have been similarly investigated, viz.:

Crystalline serum albumin, prepared from horse-serum by the method of Hopkins and Pinkus [1898].

Serum globulin (pseudo-globulin). Three different samples were investigated, obtained from horse-serum by different methods. I and II were prepared by repeated precipitation with half-saturated ammonium sulphate, the euglobulin present in the serum being separated by the subsequent dialysis; in the case of III the latter was removed by preliminary precipitation with saturated brine, after which the pseudo-globulin was precipitated from the warm diluted filtrate by adding anhydrous sodium sulphate until the concentration equalled 20 % Na_2SO_4. The precipitate was thoroughly washed with a solution of sodium sulphate of the same strength.

Crystalline egg-albumin, prepared from egg-white, also by the method of Hopkins and Pinkus.

All the proteins were thoroughly dialysed in presence of toluene and filtered, and concentrated solutions were finally obtained. Determinations were then made of the protein-content and the density. The latter were obtained by pyknometer readings, carried out usually at 15° C. or 20° C., the density of water at the same temperature being taken as unity for the purpose of calculation. It was found that the proportionality between the density of water and that of the protein solution was, within our error of experimentation, maintained between 15° C. and 25° C.

Dry specimens of the proteins were obtained by evaporating the solutions to dryness and grinding the residue to a fine powder. The evaporation and drying were carried out *in vacuo* at room-temperature, and were continued until the weight remained constant, an operation requiring 2–6 weeks. It was feared that, if the samples were dried at a high temperature, the denaturation of the proteins might introduce a source of error. This would not appear to be so, for, in the case of serum albumin, the densities of two samples dried at 100°–110° and 20° respectively, were not found to differ by any significant amount (Table I). The densities of the various powders were determined by weighing in benzene of known density (water at 4° C. = 1·00) at 25°, as described above.

TABLE I.

Density of Protein in solution compared with that in the solid state.

Protein	*In solution*			*Dry State*
	Concentration of protein % (by weight)	Density of the solution	Calculated density* of the protein	Density* of the protein
Caseinogen	7·85	1·0241	1·39†	1·318
Egg-albumin (crystalline) ...	14·6	1·0401	1·359	1·269‡
Serum-albumin (crystalline)	22·15	1·0647	1·378	1·275§
				1·281‡
Serum-globulin I	15·33	1·0428	1·365	1·279§
Serum-globulin II	16 35	1·0466	1·374	1·289‡
Serum-globulin III	11·05	1·0316	1·384	1·312‡

* Density of water at 4° C. = 1·00.
† Corrected for presence of sodium.
‡ Dried at room temperature *in vacuo*.
§ Dried at 105°—110° C.

The results are given in Table I and show that the density of albumin and globulin is also increased when in the state of colloidal solution, and to a higher degree (6·8 %) than was found for caseinogen (5 %). Egg- and serum-albumin have an almost identical density in the dry condition, viz. 1·269 and 1·281, and this is increased in the same proportion on entering solution, viz. to 1·359 and 1·378 respectively. The three samples of globulin give 1·293 as mean value when dry and 1·374 when in solution.

In Tables II and III are given the variation of the density of solutions of serum-albumin and serum-globulin with alteration in protein concentration. If the latter is plotted against the former, straight lines are obtained. The curve for caseinogen, on the other hand, shows a slight convexity which

is rather more than can be explained by our experimental error, the contraction on entering solution being proportionally greater for dilute solutions. This is shown in the third column of Table II, where the value of the density of caseinogen calculated from that of its solution is seen to decrease progressively from 1·465 to 1·412 as the concentration of the protein is increased. In the case of the serum proteins, however, the value remains constant (Table III).

TABLE II.

Density of Caseinogen (Na Caseinogenate) solutions of varying concentration.

Concentration of caseinogen % (by weight)	Density of the solution*	Calculated density of sodium caseinogenate
9·39	1·0283	1·412
8·33	1·0250	1·409
7·52	1·0232	1·424
6·05	1·0190	1·437
4·35	1·0140	1·460
2·173	1·0070	1·465
1·086	1·0033	†

* Compared with water = 1·00 at the same temperature, 15° C.
† Solution too dilute for trustworthy calculation.

TABLE III.

Density of solutions of Horse-Serum Proteins of varying concentration.

Protein	Concentration % (by weight)	Density of the solution*	Calculated density of the protein
Serum-globulin	15·33	1·0428	1·365
,, ...	10·32	1·0290	1·374
,, ...	6·916	1·0190	1·365
,, ...	3·478	1·0096	1·370
Serum-albumin	22·15	1·0647	1·378
,, ...	15·16	1·0440	1·382
,, ...	7·725	1·0220	1·381

* Compared with water = 1·00 at the temperature of expt.

SUMMARY.

A comparison has been instituted in case of four proteins, viz. caseinogen, egg- and serum-albumin, and serum globulin, between the density directly determined with dry specimens and that calculated from the specific gravity

of concentrated solutions. The latter is found to be 5–8 % in excess of the former, showing the extent of shrinkage in volume taking place when these proteins enter colloidal solution.

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IX. A NOTE CONCERNING THE INFLUENCE OF DIETS UPON GROWTH.

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(*Received December 22nd, 1912.*)

In a recent paper Osborne and Mendel [1912, 1] have described certain experiments which seem to show that young animals (rats) can grow when fed upon artificial diets consisting of "purified" constituents alone.

These experiments would therefore indicate that the accessory factors of uncertain nature (complex lipoids or "vitamines" or "hormones") which others have believed to be necessary are not, as a matter of fact, indispensable. Such experimental results must give pause to those who like ourselves are engaged in an endeavour to separate, and identify more closely, the accessory substances referred to. But they are results which contradict what is now a considerable body of experience, and the experiments which yielded them seem to call for repetition.

These particular experiments were in a sense merely incidental to a wide enquiry, on the part of the authors quoted, into the nutritive efficiency of various proteins; and those which are of significance to the matter at issue (those, namely, which showed actual growth, and not maintenance alone) concerned only three animals [1912, 1, pp. 356, 358]. In a later paper [Osborne and Mendel, 1912, 2] the authors again refer incidentally to the subject and speak of having obtained "a considerable degree of success" by feeding in the absence of "the hypothetical organic hormones," etc. [1912, 2, p. 242].

But the weight-curves given in this paper show little more than maintenance of the animal, without growth; while, for some reason, the three experiments of the earlier paper which showed complete success in the promotion of vigorous growth are not further quoted. This success the authors attributed in the main to the fact that, in this dietary, salts were

supplied in a mixture made to imitate as exactly as possible the salts of milk; but it is difficult to understand how animals so omnivorous as rats can depend for normal growth upon a very exact balance of particular acid and basic ions. In the experiments described by one of us in an earlier paper [Hopkins, 1912] the salts administered were obtained by carefully ashing a normal food mixture of proved efficiency. Upon an artificial diet containing these salts the animals did not grow; but they grew at once when certain substances were added to the diet, some of these addenda being certainly incapable of supplying any deficiencies in the inorganic constituents of the original diet.

We have now fed a large number of rats upon the diet employed by Osborne and Mendel. The salt mixture as described by them was made with the greatest care, and all their directions for the preparation of the food mixture were exactly followed. But the protein and starch were thoroughly extracted with alcohol, and the lactose used was several times precipitated from its aqueous solution by the addition of alcohol. The methods of feeding were exactly those used in the paper by one of us already referred to, except that the food, being more fatty and coherent, was not mixed with water.

Twenty-four rats from various sources, of weights from 50 to 60 grms., were placed upon the mixture. Although the consumption of food was satisfactory, every rat, without exception, rapidly ceased to grow. In the greater number growth ceased as early as the sixth day, in some on the ninth, and in all before the fifteenth day. A comparatively brief period of maintained weight was then followed by a steady decline. In the case of eighteen of the animals the diet was administered up to the time of death, which, in all but four cases, occurred before the fortieth day.

To six of the above set of rats, after the decline in their weight had begun, 2 c.c. of milk per diem were given. An immediate betterment of the general condition of the animals followed; growth was re-established and the health then maintained. In another experiment six rats were put upon Osborne and Mendel's diet, but were given milk from the first. In each case the animal grew.

We have spoken of the food consumption (which was carefully determined each day) as being satisfactory. It was smaller however than the consumption of the animals upon a somewhat different artificial dietary in the experiments described earlier by one of us. Its energy value, during the period which preceded actual loss of weight, was just under 40 calories per 100 grms. live weight, instead of over 50 calories. That the former value is nevertheless well in excess of the amount required for maintenance is shown by some, as

yet unpublished, experiments upon comparable animals made in the Cambridge Physiological Laboratory by Miss Hill.

When the small ration of milk was given each day in advance of feeding with the Osborne and Mendel diet the food consumption remained of the same order, and did not rise to the amount consumed by the rats in Hopkins' earlier experiments [1912]. The resulting growth though quite definite and steady was distinctly slower than in the experiments mentioned.

Our rats clearly behaved very differently from the three animals fed upon a similar diet by Osborne and Mendel. The difference we are unable to explain. Realising from previous experience how very small a remainder of the substances which are extracted by alcohol may leave an artificial dietary with some power of maintaining growth, and knowing that ether is a greatly inferior solvent for them, we fed rats upon the Osborne and Mendel mixture in which the protein (commercial casein) was extracted with ether only (as in one of Osborne and Mendel's experiments [1912, 1]; Curve 58, p. 358) and the lactose not crystallised from alcohol. We were unable to obtain growth however, though even this small difference in the diet appreciably lengthened the period during which the animals remained in health.

We do not in this note propose to publish the schedules of weights, etc. They will be given elsewhere at a later date. The purpose of the present note is to indicate that there is still reason for a continuance of the search for special accessory substances of potent influence upon growth. It should be pointed out that Osborne and Mendel themselves admit that such substances may exist.

REFERENCES.

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